

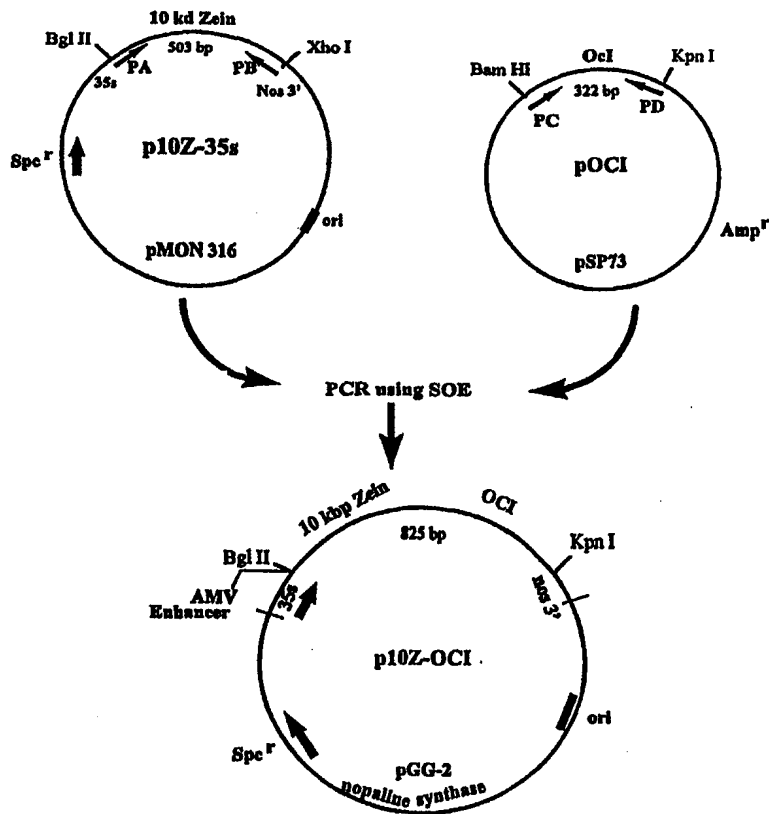


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/82, C07K 14/425, C12N 15/29, 15/62, C07K 16/16, 16/38, A01H 5/00	A1	(11) International Publication Number: WO 00/40738 (43) International Publication Date: 13 July 2000 (13.07.00)
(21) International Application Number: PCT/US99/08525 (22) International Filing Date: 16 April 1999 (16.04.99) (30) Priority Data: 09/224,655 31 December 1998 (31.12.98) US (71) Applicant: NEW MEXICO STATE UNIVERSITY TECHNOLOGY TRANSFER CORPORATION [US/US]; Box 30001, MSC 3RES, Las Cruces, NM 88003 (US). (72) Inventors: BAGGA, Suman; 415 West College Avenue #3, Las Cruces, NM 88005 (US). SENGUPTA-GOPALAN, Champa; 3059 Camino Real, Las Cruces, NM 88001 (US). KEMP, John, D.; 6760 Raasaf Drive, Las Cruces, NM 88005 (US). (74) Agents: LLOYD, Jeff et al.; Saliwanchik, Lloyd & Saliwanchik, Room A-1, 2421 N.W. 41st Street, Gainesville, FL 32606 (US).		(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>

(54) Title: CO-EXPRESSION OF PROTEINS**(57) Abstract**

The subject invention pertains to materials and methods for transformed plants and plant tissues that are capable of expressing high levels of stable proteins which are localized as protein bodies within the plant cell. Transformed plants co-expressing high levels of both the 15kD and 10kD zein proteins are disclosed which accumulate to high levels as protein bodies in the vegetative tissue of the plant. Transformed plants co-expressing the 15kD and 10kD zein proteins are useful for providing forage crops containing increased levels of sulfur containing amino acids, such as methionine, in the diet of animals that normally feed on such crops. Also contemplated by the subject invention are transformed plants or plant tissue comprising stable protein bodies which contain heterologous proteinaceous material. In one embodiment, a stable protein body is expressed in a plant or plant tissue as a fusion protein comprising a zein protein and an operably linked protein or peptide. The protein bodies provided in the present invention are resistant to rumin digestion or environmental degradation.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

DESCRIPTION
CO-EXPRESSION OF PROTEINS

Cross-Reference to a Related Application

5 This is a continuation-in-part application of co-pending application Serial No. 08/866,879, filed May 30, 1997; and provisional application Serial No. 60/020,424, filed May 31, 1996, now abandoned.

Background of the Invention

10 Alfalfa (*Medicago sativa* L.) is considered to be the most important cultivated forage crop in the world (Hanson *et al.*, 1988; Michaud *et al.*, 1988) and is often referred to as "Queen of the forage crops" because it is widely grown, has a superb balance of vitamins and minerals, is high yielding, is an excellent source of biological nitrogen fixation, and it serves as an attractive nectar source for honeybees (Barnes *et al.*, 1988; Smoliak and Bjorge, 1983). Alfalfa
15 has been bred for years for both forage quality and plant performance. Although alfalfa and other leguminous forage crops are high in protein, these plants are deficient in the sulfur amino acids (S-amino acids), methionine and cysteine (Kaldy *et al.*, 1979). It has been shown that wool growth in sheep is limited by the availability of S-amino acids. Similarly, milk production by dairy animals is affected by the deficiency of S-amino acids in plants. Efforts to use
20 conventional plant breeding and cell selection techniques to increase the S-amino acid content of alfalfa have met with little or no success.

 A genetic engineering approach to improve the amino acid balance of alfalfa and other forage crops would be to introduce into these plants genes encoding proteins high in methionine driven by a strong constitutive promoter or a leaf promoter. In order to significantly alter the
25 amino acid balance of legume forage, the foreign proteins should contain about 15 to 25% of S-amino acids and constitute 5 to 10% of the total leaf protein. To achieve these levels of protein accumulation, one has to ensure not only maximum levels of transcription and translation of the gene but also the stability of the protein. In regard to forage crops for ruminant animals, the digestibility of S-amino acid containing proteins by the rumen bacteria and the stomach enzymes
30 is also an extremely critical issue in regard to providing a suitable forage crop for ruminant animals, but is often overlooked. Thus, the S-amino acid rich protein should be relatively resistant to degradation in the rumen (first stomach) of the ruminant animals and should be assimilated in the lower gastrointestinal tract.

 Most of the concerted efforts in regard to nutritional improvement in plants has focused

on seed proteins. Since corn and other cereal crops are not easily transformable, most work directed to seed protein modification has involved testing stability of modified prolamine proteins in transgenic tobacco (Williamson *et al.*, 1988; Ohtani *et al.*, 1990) and *Xenopus oocytes* (Wallace *et al.* 1988). The synthesis of lysine containing α zeins was also analyzed in transgenic tobacco and petunia seeds (Williamson *et al.*, 1988; Ohtani *et al.*, 1990). Both the normal and modified protein were found to have a very short half-life.

Efforts to improve the S-amino acid content of legume seed proteins have included introducing a 45bp oligonucleotide containing six methionine codons into the third exon of a β -phaseolin gene. Transformants containing this modified gene showed that the high methionine phaseolin was synthesized at the same level as the normal protein, but was very unstable and was rapidly turned over (Hoffman *et al.*, 1988). Introduction of the extra amino acids in the β -phaseolin protein probably caused a distortion in its secondary structure making it more susceptible to proteolytic degradation. DeClercq *et al.* (1990), replaced a 23 amino acid coding segment between the sixth and seventh cysteine residues of Arabidopsis 2S albumin, with three different high methionine coding fragments. These modified Arabidopsis 2S genes were transformed into *A. thaliana*, *B. napus* and tobacco. There was some accumulation of the protein in the seeds but not as much as predicted. (Chrispeefs, M., personal communication). The gene of the 2S albumin of Brazil nut, which contains up to 19% methionine, and driven by the β -phaseolin gene promoter, has been introduced into tobacco (Guerche *et al.*, 1990), rape (Altenbach *et al.*, 1992) and soybean (Pioneer Seed Co.). Recently, Saalbach *et al.* (1994) synthesized the 2S albumin gene and engineered it behind the CaMV 35S promoter. The gene, when introduced into tobacco and some grain legumes, showed the highest level of expression in the plant leaves and the protein was localized in vacuoles. However, the Brazil nut albumin protein is extremely allergenic and may not be acceptable for consumption.

One approach to increase the pools of particular amino acids in plants has been to introduce bacterial genes encoding for key regulatory enzymes in amino acid biosynthetic pathways in plants. A bacterial gene encoding for aspartate kinase which is desensitized to feedback inhibition by lysine and threonine was fused to the β -phaseolin gene promoter and introduced into tobacco. The seeds of the transgenic tobacco showed increased levels of free threonine and methionine (Karchi *et al.*, 1993; Galili, 1995).

Very little effort has been made with regards to improving forage crop protein quality. Schoeder *et al.*, (1991) introduced the chicken ovalbumin gene (cDNA), driven by a CaMV 35S promoter, into alfalfa. The transgenic alfalfa plants, however, showed very low level accumulation of the protein in the leaves (0.005%). The basis for such a low abundance of this

protein in the transgenic alfalfa leaves was not determined.

Some efforts to obtain alfalfa mutants that have larger free methionine levels have also been attempted at the University of Wisconsin. Cell lines with resistance to growth inhibition by an amino acid analogs reportedly produce higher than normal amounts of the corresponding natural amino acid. Hence, growth on specific amino acid analogs has been used as selection
5 tool to select for plants accumulating high levels of a particular amino acid. Amino acid over-production is usually due to relaxed feedback control of an enzyme involved in its production (Malega, 1978). In an attempt to improve the methionine content of alfalfa, mutagenized suspension culture cells of alfalfa were selected for resistance to growth inhibition by a
10 methionine analog (Reish *et al.*, 1981). A few cell lines containing high methionine pools were obtained, however, regeneration of these cell lines did not produce plants with high methionine content (personal communication, Bingham, ET).

Zeins are a group of alcohol soluble proteins that are synthesized during endosperm development in corn and constitute 50% of the total protein in mature seeds (Lee *et al.*, 1976).
15 The zeins can be divided into four groups, the α , β , γ and δ , based on their solubility (Larkins *et al.*, 1989). The zeins can also be separated by size into groups. The α zeins, which is the most abundant class, are made up of the 22kD and 19kD zeins; the central region of these proteins consists of repetitive peptides of about 20 amino acid residues (Argos, 1982). The β zeins comprise the 15kD zein which contains less proline and glutamine than the α zeins. The
20 γ zeins include the 27kD and 16kD class and are very rich in proline (25%). The δ zeins are a relatively minor class consisting of the 10kD zein (Kirihaara *et al.*, 1988). All the zein classes are structurally unique. The repeat regions in the α and γ zeins probably have a major role in the packing of protein bodies. Zeins, in general, contain extremely low levels of the essential amino acids lysine, tryptophan and to a lesser extent methionine. The 15kD and 10kD zeins,
25 however, are distinguished by their extremely high methionine content (10% and 22.5%, respectively) (Giannaza *et al.*, 1977).

The zeins are synthesized on the rough endoplasmic reticulum (RER) and they aggregate into protein bodies directly in the RER (Larkins and Hurkman, 1978). Based on the analysis of the zein composition of developing protein bodies in corn endosperm, Lending and Larkins
30 (1989), have proposed a descriptive model for the pattern of zein deposition during protein body formation in corn endosperm: The β and γ zeins are the first to start accumulating within the RER. Subsequently, α zeins begin to accumulate as locules within the β and γ zeins. With time, the α zein locules fuse and form a central core while the β and γ zeins form a continuous layer around the periphery of the protein body. In a separate study, Esen and Stetter (1992),

demonstrated that the δ zein occurs throughout the core region of the protein body.

Mutations in maize affect the expression of the different zein genes. Changes in zein gene expression in turn have direct impact on the amino acid composition of the seeds. Seeds of plants homozygous for the recessive mutation opaque-2, have increased levels of lysine compared to the wild-type seeds (Misra *et al.*, 1972). The increase in lysine is due to the reduced expression of the 22kD α zeins (Langridge *et al.*, 1983). The inbred line BSSS-53 has 30% higher level of seed methionine compared to other inbred lines. This increase in methionine content is because of a two-fold increase in the level of the 10kD zein (Phillips and McClure 1985).

Proteins that accumulate in the endoplasmic reticulum are known to have the amino acid sequence Lys(his) Asp Glu Leu (K(H)DEL) near their carboxy terminal end which prevents them from exiting into the Golgi (Pelham, 1990). The zeins and other prolamines, however, lack this sequence. A cognate of the 70-kD heat shock protein, BiP which functions as a molecular chaperone has been shown to be involved in the formation of prolamine protein body formation in rice endosperm (Li *et al.*, 1993b). The involvement of BiP in the formation of zein protein bodies is based on the fact that BiP accumulates to high levels in the ER and on the abnormal protein bodies of some of the zein regulatory mutants of corn (Boston *et al.*, 1991; Zhang & Boston, 1992). Overall, however, the mechanisms of zein targeting and assembly in protein bodies are poorly understood and it is not known whether inter- and intra-molecular interactions play a key role in protein body formation. Abe *et al.* (1991), have suggested that the cytoskeleton plays a role in the biogenesis of zein protein bodies.

As can be understood from the above, there remains a need in the art for plants and forage crops that contain stable protein bodies that are high in S-amino acid content. The subject invention provides a novel and advantageous means for improving the forage quality of plants.

Brief Description of the Drawings

Figures 1A-1B show a steady-state accumulation pattern of the 15kD zein protein in leaves of transgenic *L. japonicus* (panel A) and alfalfa Regen SY (panel B). 70% ethanol (EtOH) soluble protein (equivalent to 50 μ g of the phosphate buffered saline soluble fraction) from leaves of different independent transformants was subjected to SDS-PAGE, electroblotted onto nitrocellulose and followed by immunoblot analysis using the 15kD zein antibody.

Figure 1C shows a diagrammatic representation of the 15kD zein gene construct.

Figures 2A-2B show a steady-state accumulation pattern of the 10kD zein protein in leaves of transgenic *L. japonicus* (panel A) and alfalfa, Regen SY (panel B). 70% Et-OH-soluble protein (equivalent to 50 μ g of the phosphate buffered saline soluble fraction) from

leaves of different independent transformants was subjected to SDS-PAGE, electroblotted onto nitrocellulose and followed by immunoblot analysis using the 10kD zein antibody.

Figure 2C shows a diagrammatic representation of the 10kD zein gene construct.

Figures 3A-3C show a steady-state accumulation pattern of the 10kD zein in transgenic tobacco.

Figure 3A. Diagrammatic representation of pM10Z. The construct consists of the CaMV 35S promoter fused at the BgIII site to a 470 bp BgIII-XhoI fragment containing the coding region of the 10kD zein gene. This is followed by the NOS 3' terminator of pMON316 (Rogers et al., 1987).

Figure 3B. Analysis of different independent transformants for the accumulation of the 10kD zein. EtOH soluble protein extracted from the leaves (equivalent to 50 µg of PBS soluble protein), was subjected to SDS PAGE, transferred to nitrocellulose and followed by immunoblot analysis using the 10kD zein antibodies. Lanes labeled 1 through 7 (under transformants), contain samples from the leaves of different transformants while lanes 1 and 2 (under control), are leaf samples from nontransformed tobacco plants.

Figure 3C. Analysis of different plant organs (transformant 6) for the accumulation of the 10kD zein. EtOH soluble fractions (equivalent to 50 µg of PBS soluble protein) from the various plant parts indicated, along with 2 µg of corn seed protein were subjected to SDS PAGE followed by western analysis using the 10kD zein antibodies. UT and Mat stand for untransformed and mature seeds, respectively. Molecular weight standards were included in the gels and the size of two of the relevant markers is indicated.

Figure 4 shows the fate of 10kD zein in germinating seeds/seedlings of transgenic tobacco. Seeds of a 10kD zein plant were sterilized and allowed to germinate under sterile conditions and at defined times as indicated in the Figure, seeds/seedlings were harvested and the EtOH soluble fraction extracted. EtOH soluble fraction equivalent of 50 µg of PBS soluble protein was then subjected to SDS PAGE followed by western analysis using the 10kD zein antibody. The position of the 10kD zein on the gel is indicated, and DAG stands for days after germination.

Figure 5A-5D. Ultrastructure and Immunogold localization of 10kD zein in transgenic tobacco leaves.

Figure 5A. Regions of two mesophyll cells showing several 10kD zein protein bodies (indicated by arrowheads) in a 10kD zein transformant.

Figure 5B. Higher magnification of 10kD zein protein bodies (indicated by arrowheads).

Figure 5C. Structure of a 15kD zein protein body in the mesophyll cell of a 15kD zein transformant.

Figure 5D. Immunolocalization of 10kD zein in the leaf cells of a 10kD zein transformant with the 5 nm gold particles (indicated by arrowheads).

5 **Figure 6A-6D.** Comparison of 10kD and 15kD zein levels in the leaves and seeds of the 10kD, 15kD and 10kD/15kD zein plants.

Figure 6A. EtOH soluble fraction from the leaves (equivalent to 10 µg of PBS soluble protein) and seeds (equivalent to 50 µg of PBS soluble protein) of the 10kD and 10kD/15kD zein plants were subjected to SDS PAGE followed by western analysis using the 10kD zein
10 antibodies.

Figure 6B. Quantification of band intensity from Figure 6A using the Bio Image Intelligent Quantifier.

Figure 6C. EtOH soluble fraction from the leaves and seeds (equivalent to 50 µg of PBS soluble protein) of the 10kD and 10kD/15kD zein plants were subjected to SDS PAGE followed
15 by western analysis using the 15kD zein antibodies.

Figure 6D. Quantification of band intensity from Figure 6C using the Bio Image Intelligent Quantifier.

Figure 7A-7D. Subcellular localization of the 10kD and 15kD zeins in leaves of the 10kD/15kD zein plants.

20 Figure 7A. Conventionally fixed and stained sections of leaves from a 10kD/15kD zein plant. Arrowheads point to the protein bodies formed in the cytoplasm.

Figure 7B. Immunolocalization of the 10kD zein protein using mouse anti-10kD zein antibody diluted 1:50 followed by 10 nm diameter gold-conjugated goat anti-mouse IgG.

Figure 7C. Co-immunolocalization of the 10kD and 15kD zeins using the mouse anti-
25 10kD zein antibody and the rabbit anti-15kD zein antibodies followed by 10 nm diameter gold conjugated goat anti-mouse IgG and 5 nm gold conjugated goat anti rabbit IgG.

Figure 7D. A higher magnification of a region showing double-labeling from panel C. The arrowheads point to the 10 nm gold particles while the arrows point to the 5 nm gold particles.

30 **Figure 8.** Analysis of the accumulation pattern of BiP in the δ -, β -, and δ -/ β -zein tobacco transformants.

Phosphate buffered saline soluble extract (100 µg of protein) from leaves of a control (NT), δ -zein, β -zein and δ -/ β -zein plants were subjected to SDS PAGE followed by western blotting using an antibody raised to BiP from maize. A positive control lane containing purified

BiP (1 µg) was included in the gel. The lower panel represents the results of band quantification using the Intelligent Quantifier.

Figure 9A represents SEQ ID NO: 5.

Figure 9B represent SEQ ID NO: 6.

5 **Figure 10** is an overview of the construction of the Z10/OCI plasmid which was transformed into tobacco.

Figures 11A and 11B are western blots of extracted samples of transformed tobacco plants showing a positive immunoreaction with both the Z10 (14A) and OCI (14B) antibodies.

10 Brief Description of the Sequences

SEQ ID NO: 1 represents primer PA which was used to amplify a Z10 fragment from plasmid prep (993) Z10 pMON 316/DH5δ.

SEQ ID NO: 2 represents primer PB which was used to amplify a Z10 fragment from plasmid prep (993) Z10 pMON 316/DH5α.

15 **SEQ ID NO: 3** represents primer PC which was used to amplify a OC-1 fragment from plasmid (809) OclpSP73/DH5α.

SEQ ID NO: 4 represents primer PD which was used to amplify a OC-1 fragment from plasmid (809) OclpSP73/DH5α.

SEQ ID NO: 5 represents a Z10 fragment as illustrated in Figure 9A.

20 **SEQ ID NO: 6** represents an OCI fragment as illustrated in Figure 9B.

Brief Summary of the Invention

The present invention relates to the co-expression of proteins that results in a greater accumulation in a cell of one of the proteins when compared to the levels of the same protein
25 when expressed alone. The proteins are expressed in prokaryotic or eukaryotic cells. One or both of the proteins are accumulated in the cell at higher levels when expressed together than when the proteins are expressed alone in a cell. Standard, routine genetic engineering techniques are employed to (i) isolate the appropriate DNA encoding desired proteins including regulatory sequences, (ii) transform a target cell and, in the case of plants, to regenerate a transgenic plant.
30 The transgenic cells are grown under conditions sufficient to result in the accumulation of one or both proteins at high levels. The proteins accumulated in the cell exhibit increased stability and resistance to degradation.

The subject invention also concerns plants and plant tissues that are capable of expressing high levels of stable proteins which are localized as protein bodies within the plant

cell. Specifically exemplified are plants co-expressing both the 15kD and 10kD zein proteins. Transformed plants co-expressing the 15kD and 10kD zein proteins are useful for providing forage crops containing increased levels of sulfur containing amino acids, such as methionine, in the diet of animals that normally feed on such crops. Also contemplated by the subject invention are plants or plant tissues containing novel heterotypic protein bodies elevated in one or more other essential amino acids (e.g., arginine, histidine, leucine, isoleucine, lysine, phenylalanine, threonine, tryptophan, tyrosine and valine). Also contemplated by the present invention are protein bodies which result in the enhanced accumulation of normally unstable proteins in plant tissue.

10 The subject invention also concerns plants or plant tissue comprising rumin stable protein bodies which contain other proteinaceous material, for example, an antigenic determinant capable of eliciting an immune response, a proteinaceous drug, pesticide or antimicrobial peptide. Heterologous proteins can be expressed in plants transformed with the storage proteins of the subject invention which can act as a "carrier protein," whereby the proteins coalesce and
15 accumulate in the cell as a protein body. In an alternative embodiment, a rumin stable protein body is provided in a plant or plant tissue as a fusion protein expressed in the cell comprising a zein protein and a heterologous protein or peptide.

Detailed Description of the Invention

20 The subject invention concerns plants and plant tissues that are capable of expressing high levels of stable storage proteins which are localized as protein bodies within the plant cell. Plants contemplated within the scope of the invention include forage crop plants, including, for example, alfalfa, clover, corn silage, sorghum and other leguminous crops, transformed to express the proteins of the invention. Also contemplated within the scope of the present
25 invention are plants for human consumption which have been transformed to express proteins that enhance the protein quality of the plant for improved nutrition. Specifically exemplified are plants expressing proteins containing high levels of S-amino acids, such as methionine and cysteine. In a preferred embodiment, a zein protein is expressed in the plant or plant tissue. More preferably, the zein protein expressed is the 15kD or 10kD zein protein. Most preferably,
30 both the 15kD and 10kD zein proteins are co-expressed in the plant or plant tissue. Plants having genotypes carrying the 10kD and 15kD zein genes were sexually crossed to create hybrids carrying both constructs. The zein proteins expressed in the transformed plants are resistant to rumin degradation and, therefore, are useful for providing nutritionally important amino acids that can be digested in the stomach and absorbed by the ruminant animal because

of the protein's capacity to "by-pass" the rumin.

Also contemplated by the subject invention are plants or plant tissue comprising rumin stable protein bodies which contain other proteinaceous material, for example, an antigenic determinant capable of eliciting an immune response, a proteinaceous drug, pesticide or antimicrobial peptide. Heterologous and endogenous proteins and synthetic peptides having essential amino acids can be expressed in plants transformed with the storage proteins of the subject invention which can act as a "carrier protein," whereby the proteins coalesce and accumulate in the cell as a protein body. In an alternative embodiment, a rumin stable protein body is expressed in a plant or plant tissue as a fusion protein comprising a zein protein and a heterologous protein or peptide. The fusion protein can be designed to yield the heterologous protein portion by cleavage with a selected enzyme or under certain physiological conditions. Preferably, the zein protein expressed as part of the fusion protein is the 15kD or 10kD zein protein. More preferably, both the 15kD and 10kD zein proteins are co-expressed in the plant or plant tissue comprising the fusion protein.

The subject invention also pertains to a rumin stable protein body. Rumin stable protein bodies of the invention are not subject to digestion by rumin bacteria in the rumin of an animal but can be digested proteolytic enzymes of an animal's stomach. A rumin stable protein body of the present invention can be prepared which contains heterologous proteinaceous material in addition to the rumin stable protein. For example, an antigenic determinant capable of eliciting an immune response, a proteinaceous drug, pesticide or antimicrobial peptide. Rumin stable protein bodies can be isolated from plants that have been transformed with polynucleotide molecules encoding the desired rumin stable proteins. Plant cells expressing the polynucleotide molecules encoding the desired rumin stable proteins can be readily selected and regenerated into plants or plant tissue using standard techniques known in the art.

In one embodiment of the present invention, a storage protein gene is co-expressed in a cell with a second protein gene whereby the second protein is accumulated in the cell at a level that is higher than when the second gene is expressed alone in the cell, *i.e.*, in the absence of the storage protein gene. In a preferred embodiment, the storage protein gene is a seed storage protein gene, the target cell is a plant cell and the second protein gene can be any gene encoding a desirable protein. Regulatory sequences employed with the protein genes (promoters, initiation sequences, termination sequences, polyadenylation sequences, enhancers, etc) are readily chosen by one of ordinary skill in the art based on a variety of factors, such as, for example, i) the specific protein genes employed, ii) the target cell to be transformed, iii) the plant tissue where expression/accumulation is desired, iv) the particular plant (monocot, dicot,

etc) species to be transformed, etc. For example, when a plant cell is the target cell then a constitutive promoter may be chosen (*e.g.*, CaMV 35S, ubiquitin, etc) or a tissue specific promoter may be employed that will express at high levels in specific tissues (seeds, green tissues, etc).

5 In another embodiment of the present invention, a 15 kD zein protein gene is employed with a second protein gene in a plant cell that results in accumulation of the second protein in the plant cell. The genes can be contained on a single expression cassette and inserted into the plant genome employing standard transformation and regeneration techniques. Alternatively, the protein genes can be inserted into a plant cell genome independently in separate expression
10 cassettes and transgenic plants can be regenerated therefrom. Also, the protein genes can be inserted into separate plant cells and regenerated into fertile, transgenic plants each containing one of the protein genes. These transgenic plants can then be cross fertilized employing standard plant breeding techniques to result in a cross that contains both the 15 kD zein protein gene and the second protein gene wherein the second protein is accumulated in one or more plant tissues.

15 In a preferred embodiment of the present invention, alfalfa, tobacco or other plant cells are transformed with a 15 kD zein protein gene and a 10 kD zein protein (second protein) wherein both genes are driven by a constitutive promoter. Fertile, transgenic plants containing both gene constructs are regenerated. Progeny plants are grown and the 10 kD zein protein is accumulated in green tissue at level of 5 to 10 times or more when compared to the accumulation
20 level of the 10 kD protein when expressed alone.

Additionally, the present invention encompasses novel protein bodies formed as a result of expressing a storage protein gene and a second protein gene in green plant tissues. In one embodiment, the novel protein body comprises a 15kD zein protein and a second protein. The protein body is typically located in leaf tissue. In a preferred embodiment, the novel protein
25 body is located in leaf tissue and comprises a 15 kD zein protein and a 10 kD zein protein.

The subject invention also concerns a method for increasing the forage quality of a plant comprising transforming a plant or plant tissue with a polynucleotide molecule that encodes a storage protein of the present invention. Methods for transforming plants and selecting for expression of the transformed genotype are known in the art. In a preferred embodiment of the
30 method, the polynucleotide encodes a zein protein which is expressed in the plant or plant tissue. More preferably, the zein protein expressed is the 15kD or 10kD zein protein. Most preferably, both the 15kD and 10kD zein proteins are co-expressed in the transformed plant or plant tissue. Transgenic plants can be readily prepared from the transformed plant or plant tissue using standard techniques known in the art.

The subject invention also concerns methods for increasing the stability and storage of a heterologous protein in a plant or plant tissue. Heterologous proteins can be expressed in plants transformed with the storage proteins of the subject invention which can act as a "carrier protein," whereby the proteins coalesce and accumulate in the cell as a protein body. In an alternative embodiment of the subject method, a plant is transformed with a polynucleotide molecule that encodes a fusion protein comprising a storage protein of the present invention operably linked with a heterologous protein or peptide.

The zein proteins of the present invention include not only those proteins having the same amino acid sequence as found in nature, including allelic variants, but also includes those variant zein proteins having conservative amino acid substitutions, additions and deletions in the protein sequence, as long as the variant protein retains substantially the same relevant biological activity as the native zein protein. The skilled artisan, having the benefit of the teachings disclosed herein, can readily determine whether a variant protein retains the substantially the same biological activity as the non-modified protein.

15

Materials and Methods

Recombinant DNA Techniques

Standard procedures were used for recombinant DNA manipulations (Maniatis *et al.*, 1982). Plasmid pMZEI10k containing the 10kD zein cDNA isolated from a corn endosperm cDNA library (Kirihaara *et al.*, 1988), was a gift from Dr. J. Messing. A 470bp *EcoRI/XbaI* fragment containing the entire coding region was removed from pUC 119 and cloned into the *EcoRI* and *XbaI* sites of pSP73. The stop codon for the 10kD zein is contained within the *XbaI* site. The 10kD zein gene was then recovered as a *BglIII/XhoI* fragment and inserted into the *BglIII* and *XhoI* sites in the polylinker of pMON316 (Rogers *et al.*, 1987). The translation terminator following the stop codon of the 10kD zein is the NOS terminator. The resulting plasmid was called pM10Z (Fig 1A). Plasmid pMEZ is as described by Bagga *et al.* (1995).

Plant Transformation and Regeneration

The plasmid pM10Z was mobilized from *E. coli* DH5 α into the *Agrobacterium tumefaciens* receptor strain pTiT37ASE by triparental mating (Rogers *et al.*, 1987). *Nicotiana tabacum* cv Xanthi was transformed by the leaf disc procedure (Horsch *et al.*, 1987). Transformants were selected and regenerated on MS media containing 100 μ g of kanamycin/ml shoots appeared within 4-6 weeks after inoculation. The shoots were rooted on the same media without hormones and transferred to the soil.

To obtain the 10kD zein/15kD zein plants containing both the zein genes driven by the CaMV35S promoter, tobacco transformants containing either the pM10Z or pMEZ were crossed and the seeds obtained were germinated on media containing 200 µg/ml of kanamycin. Western analysis was performed with protein extracts from the seedlings using both 15kD zein and 10kD zein antibodies. Plants expressing both the zein genes (10kD/15kD zein plant) and the parent plants (10kD zein and 15kD zein plants) were used in all comparative analysis.

Zein Extraction and Western Analysis

Plant tissues were ground and extracted in phosphate buffered saline (PBS) and centrifuged. The supernatant was used for protein determination using the Bradford assay (BIO RAD). The pellet from centrifugation was incubated in 70% ethanol containing 1% of mercaptoethanol at 65°C for 30 minutes to extract the zein proteins. For western analysis, the EtOH-extractable fraction equivalent of a known amount of PBS soluble protein extract was subjected to SDS-PAGE (Laemmli, 1970), followed by electroblotting onto nitrocellulose membrane. The membrane was blocked for 1 to 2 hrs with 1% BSA in Tris-buffered saline containing 0.05% Tween 20 (TBST), followed by overnight incubation in the same solution containing the appropriate antibodies. The 10kD zein monoclonal antibodies were provided by DEKALB Genetics Corporation, the 10kD zein polyclonal antibodies by Dr. J. Messing and the 15kD zein polyclonal antibodies by Dr. B. Larkins. The protein bands reacting with the antibodies were made visible by using an alkaline phosphatase-linked second antibody (goat antibody or rabbit IgG in case of the polyclonal antibody or mouse IgG in the case of the monoclonal antibody) and the substrates, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate according to the manufacturer's instructions (Promega). Both the polyclonal and monoclonal antibodies for the 10kD zein gave similar results on western analysis and immunolocalization on the 10kD zein plants, but the polyclonal antibodies showed some degree of cross-reactivity with the 15kD zein and as such in all our comparative analysis involving the parents and the crossed progeny, only the monoclonal antibodies were used.

Analysis of BiP

The phosphate buffered saline (PBS) soluble extracts from the leaves of the different plants were subjected to SDS PAGE followed by western analysis using the polyclonal antibodies for maize BiP (provided by Dr. R. Boston) using the procedure described in zein extraction and western analysis section above.

In vivo labeling of leaf discs

Four leaf discs (7 mm in diameter) from young expanding leaves were incubated in 120 µl of labeling mix (1 mM potassium phosphate, pH 6, 1% sucrose and 50 µg of chloramphenicol) containing 120 µci of 35S methionine (specific activity 1047 ci/mMol), for 2 hours in the light. The discs were then washed well with the incubation buffer and the samples ground in cold PBS. The samples were extracted in PBS, protein estimation made in the PBS soluble fraction and the zeins were recovered from the pellet as described by Bagga *et al.* (1995). The proteins were analyzed by SDS PAGE followed by electroblotting on PVDF membrane (Millipore). The membrane was sprayed with Enhance (NEN), air dried and exposed to X-ray film.

Electron Microscopy

Small pieces of leaf and seed tissue were fixed in 2.5% glutaraldehyde in 0.07 M sodium cacodylate buffer for 2 h and then postfixed in 1% aqueous osmium tetroxide for 1 hr. The samples were dehydrated in EtOH and embedded in Spurr's resin at 70°C. Silver sections on copper grids were then stained in uranyl acetate and Reynold's lead citrate. The grids were examined in a Hitachi H7000 transmission electron microscope.

Immunoelectron Microscopy

Small pieces of leaf and seed tissue were fixed for 2 h on ice in 4% paraformaldehyde and 0.6% glutaraldehyde in 0.33 M sodium/potassium phosphate buffer (pH 7.3) containing 0.1 M sucrose. The tissue was washed in three changes of buffer containing 7% sucrose and kept in the last change overnight at 4°C. Two different protocols were used at this stage: the fixed tissue was dehydrated in EtOH, infiltrated with Lowicryl at -10°C and resin was polymerized under UV light at -10°C for 24 hr and then at room temperature for 24 hr. In the second protocol the tissue was dehydrated in EtOH and embedded in either Spurr's resin or LR White resin and polymerized at 50°C. The remaining steps were all done at room temperature. (The different resins were purchased from Electron Microscopy Sciences, Ft. Washington, PA.) Silver sections on nickel grids were first incubated in a blocking solution of 10 mM Tris-saline containing 1% BSA (Sigma), 0.05% Tween 20 (Sigma), and 15 mM NaCl. This buffer mixture was used in all of the remaining steps. For immunolabeling seed sections, 5 to 20% normal serum from the animal source of the antibody was added to the blocking solution in order to reduce non-specific staining.

Immunolabeling with the 10kD zein antibodies: The grids of sections from the 10kD

and 10kD/15kD zein crosses were drained and incubated with the monoclonal antibody to 10kD zein diluted 1:50 in buffer for 45 min to 4 h. Controls were incubated in nonimmune mouse IgG. They were then washed in the buffer and placed in gold-labeled, goat anti-mouse IgG with a diameter of 10 nm (Sigma) diluted 1:50 in buffer for 45 min. In case of the sections from the 10kD zein plant, the grids were incubated with the polyclonal rabbit anti-10kD zein diluted 1:1000 in buffer for 60 min. They were then washed and incubated in a solution of gold-conjugated anti-rabbit IgG with a diameter of 5 nm, diluted 1:50 for 60 min. Since the polyclonal 10kD zein antibody cross-reacted with the 15kD zein, in case of the 10kD/15kD zein cross, the grids were incubated with the 10kD zein monoclonal antibody followed by gold-conjugated anti-mouse IgG with a diameter of 10 nm. The grids were washed in Tris-Saline containing Tween 20 and 1% BSA, followed by double-distilled water. The grids were then examined either unstained or lightly poststained in uranyl acetate and lead citrate.

Double-labeling with the 10kD and 15kD zein antibodies: The grids were incubated in rabbit anti-15kD zein diluted to 1:100 for 45 to 60 min. The grids were washed and incubated in gold-conjugated goat anti-rabbit IgG with a diameter of 5 nm, diluted 1:50 for 45-60 min. The grids were then thoroughly washed in the buffer and then incubated with mouse anti-10kD zein diluted 1:50 in buffer for 45 to 60 min. They were then washed in buffer followed by distilled water and incubated in gold-conjugated goat anti-mouse IgG with a diameter of 10 nm diluted 1:50 in buffer for 45 min. The grids were washed in Tris-Saline containing Tween 20 and 1% BSA, followed by double-distilled water. The grids were then examined either unstained or lightly poststained in uranyl acetate and lead citrate.

Following are examples which exemplify certain embodiments of the subject invention. These examples are illustrative and should not be construed as limiting the subject invention in any manner.

Example 1 – Accumulation of the 15kD and 10kD zeins in vegetative tissues of *L. japonicus* and alfalfa

15kD and 10kD zein coding sequences under the control of a CaMV 35S promoter were introduced into *L. japonicus* and alfalfa (Regen SY). As shown in Figures 1 and 2, both zein proteins showed high levels of accumulation in the vegetative tissues of all the transformants (1 to 2% of total protein). In contrast, the β -phaseolin gene driven by the CaMV 35S promoter, showed constitutive accumulation of the transcripts but, only seed specific accumulation of the protein. The results suggest that the zeins are stable in vegetative tissues while β -phaseolin,

which is a vacuolar protein, is not. The stability of the zein proteins can be attributed to the intrinsic properties of the protein or to its subcellular location.

Example 2 – Accumulation of zein protein in vegetative tissues of tobacco transformants

5 Tobacco plants were transformed with the gene construct pM10Z, consisting of the 10kD zein gene driven by the CaMV 35S promoter (Figure 3A). Leaves from seven randomly picked independent transformants were subjected to western analysis to measure the accumulation of 10kD zein (Figure 3B). All of the transformants showed two immunoreactive proteins, one of which co-migrated with the 10kD zein from corn seeds (position indicated by arrow) and the
10 other as a 29kD band. The latter did not co-migrate with the higher molecular weight immunoreactive band seen in corn seeds (Figure 3C). The 29kD immunoreactive band obtained from the leaves of the transgenic plants probably represents an aggregate of the 10kD zein and another endogenous leaf protein. Similarly, the 20kD immunoreactive protein band in the corn seed may represent an aggregate of the 10kD zein with an endogenous corn protein. The
15 accumulation of the 10kD and the 29kD immunoreactive bands differed by about 10 fold among the different transformants. Transformant 4 showed almost negligible level of both the immunoreactive bands. Differences in the amount of accumulation of the 10kD zein in the various transformants can probably be attributed to position effect or the number of copies of the integrated gene.

20 To determine the accumulation of 10kD zein protein among different plant parts, equal amounts of protein extract (equivalent of 50 µg of PBS soluble protein) from the leaf, stem, root and seeds of transformant 6 were subjected to western analysis along with corn seed extract (equivalent of 2 µg PBS soluble fraction) (Figure 3C). The leaves showed the highest level of accumulation of 10kD zein followed by the stem. The seeds had about a 10-fold lower level of
25 10kD zein compared to leaves, as was the case with the 15kD zein in transgenic tobacco (Bagga *et al.*, 1995). Taken together, our results suggest that 10kD zein accumulates to significant levels in all organs of transgenic tobacco, as we had reported earlier for 15kD zein (Bagga *et al.*, 1995).

30 Example 3 – Stability of zein in germinating tobacco seeds

It was previously shown that the 15kD zein in transgenic tobacco seeds is not proteolytically digested during germination (Hoffman *et al.*, 1987; Bagga *et al.*, 1997). To determine if the 10kD zein behaves in a similar manner, seeds from a 10kD zein plant were allowed to germinate for different time periods (0 to 10 days), the seeds/seedlings harvested and

their ethanol-soluble proteins extracted and analyzed by western analysis using the 10kD zein antibodies (Figure 4). The level of 10kD zein remained essentially unchanged for the first four days of germination, after which the level showed a dramatic increase in concentration. A slight drop in the level of the 10kD zein was observed between 0 and 1 day after germination (DAG), but after that the level was maintained till 4DAG. The drop observed with the 3DAG sample was not consistent within different experiments and is attributed to lower load of the protein extract in that lane. The 4DAG time point coincided with the appearance of the first set of green leaves and may be related to the activation of the CaMV 35S promoter in the developing seedling. The immunoreactive 10kD zein band also appeared fairly diffused in SDS-gels of proteins from the seedling stage, as has been observed with the leaf sample, suggesting that the leaves have some material in the ethanol-soluble protein fraction that interferes with the mobility of 10kD zein. These results suggest that 10kD zein is not degraded during germination of tobacco seeds.

Example 4 – Immunolocalization of the 15kD and 10kD zein proteins in novel protein bodies in transgenic plants

To understand the basis for the stability of the 15kD and 10kD zein proteins in the leaves of transgenic plants, the location of these proteins at a subcellular level was examined. Leaves of the transformants, along with control plants, were subjected to ultrastructural analysis followed by immunocytochemistry (using 15kD and 10kD zein antibodies). The 15kD zein protein appears to be uniformly distributed in unique rosette-shaped protein bodies lined by RER. (Bagga *et al.*, 1995, Appendix). These protein bodies were also seen in *L. japonicus* and alfalfa expressing this 15kD zein gene.

Electron microscopy was also performed on leaf tissue from transgenic tobacco expressing the 10kD zein to check for the presence of any protein bodies. As seen in Figure 5A and 5B, protein bodies very different from the 15kD zein protein bodies (Figure 5C) were seen in the leaves of the 10kD zein plants. The protein bodies in the 10kD zein plants appeared very osmophilic, the osmophilia being concentrated along the circumference of the bodies. In some cross sections, the osmophilia appeared to radiate in discrete spokes from a central hub (Figure 5B). The protein bodies seen in the 15kD zein plants did not exhibit this extreme osmophilia (Figure 5C). In some of the leaf sections, the 10kD zein protein bodies were found to be associated with the ER but in most cases because of the large size of the bodies the ER membranes appeared disjointed. Based on immunolocalization, the 10kD zein was found to be evenly distributed in these unique protein bodies, suggesting that they result from assembly of

the 10kD zein (Figure 5D).

Example 5 – Simultaneous accumulation of the 15kD and 10kD zeins in transformants expressing both genes

5 Sexual crosses were made between tobacco transformants expressing either the 15kD or 10kD zeins in order to determine if these proteins interact with one another and affect protein accumulation in the plant cell. Seeds from these crosses were germinated on 200 µg/ml of kanamycin and seedlings expressing both genes were selected based on positive PCR using both 15kD and 10kD zein gene-specific primers. Protein extracts from the leaves of two independent
10 plants expressing both genes and their respective parents were analyzed by immunoblotting using both 15kD and 10kD zein antibodies. The accumulation of the 15kD zein protein appeared similar in both the parents and the 10kD/15kD zein crossed plants, while the accumulation of the 10kD zein was many fold higher in the 10kD/15kD zein crossed plants compared to the corresponding 10kD zein parent.

15 To determine the exact level of increase of the 10kD zein due to co-expression with the 15kD zein, equal amounts of the protein extracts from the leaves and seeds of one of the 10kD zein plants, a 15kD zein plant and the corresponding 10kD/15kD zein cross was analyzed by western analysis followed by quantitation of the immunoreactive bands using an Intelligent Quantifier (BioImage) (Figure 6). This quantitative analysis showed that the 15kD zein levels in
20 both the seeds and the leaves of the 10kD/15kD zein plants were essentially similar to those in the 15kD zein parent plant (Figure 6C, 6D). However, the amount of 10kD zein, was four- to five-fold higher in the leaves and seeds of the 10kD/15kD zein cross compared to the parent plant (Figure 6A, 6B). The level of the 15kD and 10kD zein in the cross or the parental lines cannot be compared directly with each other because of differences in the antigenicity of the two
25 antibodies and the concentration of the antibodies used for developing the blots. These results also confirm previous studies indicating that the leaves accumulate more of the zein proteins than the seeds. Note that in case of the 10kD zein protein (Figure 6A, 6B), the amount of protein loaded on the gel is 10 µg for the leaf samples and 50 µg for the seed samples.

30 Example 6 – 10kD zein protein is localized in the same ER-derived protein bodies as the 15kD zein in plants co-expressing the 10kD and 15kD zein genes

Increased accumulation of the 10kD zein in the 10kD/15kD zein cross compared to the 10kD zein plant alone is suggestive of some kind of interaction between the 10kD and 15kD zeins. Electron microscopy and immunocytochemistry of leaf tissue from a 10kD/15kD zein

plant revealed only the ER-derived protein bodies typical for 15kD zein (Figure 7A). We did not observe any protein bodies similar to detected in the 10kD zein plants. However, immunolocalization of 10kD zein showed that the protein was exclusively confined in the 15kD zein protein bodies (Figure 7B). To determine whether the 10kD zein and 15kD zein were both located in the 15kD zein protein bodies, we performed double-labeling immunocytochemistry on leaf and seed sections of the 10kD/15kD zein plant using monoclonal antibodies for the 10kD zein and polyclonal antibodies for the 15kD zein. Both the 10kD zein (represented by the larger 10 nm gold particles) and 15kD zein (represented by the smaller 5 nm gold particles) were immunolocalized in the same 15kD zein protein bodies (Figure 7C,D). Thus, the 15kD and 10kD zeins have been demonstrated to interact with one another and this interaction stabilizes the two proteins.

Example 7 – Introduction of multiple copies of 15kD and 10kD zein genes into alfalfa

Multiple copies of the 15kD and 10kD genes can be introduced in plants as a method of increasing the total leaf content of S-amino acid containing proteins. In addition to those constructs utilizing the 35S promoter, gene constructs driven by the SSU promoter and the mannopine synthase promoter can be used to avoid any potential problems of co-suppression. Isogenic populations carrying either the 10kD, the 15kD, or both the 10kD and the 15kD zein constructs can be sexually developed. Trends in zein dosage effects on expression “per se” in alfalfa, interactions between constructs, and the influence of each construct on plant development, forage quality and yield can be determined based on general comparisons of the average number of zein constructs expected within a population. Genetically defined genotypes from three populations carrying from one to four copies of either the 10kD or 15kD construct or one to two copies of both the 10kD and 15kD constructs can also be examined. The most direct approach to sexually increase zein copy number among regenerated somaclones would be to either self-pollinate individual regenerates or to intercross them. Intercrossing regenerants, however, is genetically equivalent to selfing in this case. To minimize the confounding effects of inbreeding depression a series of hybrid populations can be developed to examine the influence of zein constructs on forage yield and quality of alfalfa.

Example 8 – Ruminant digestion of proteins

In ruminant animals, food is first acted upon by microorganisms inhabiting the first stomach (rumen) of the animal. The cellulose in plant material is digested by the inhabiting microorganisms since these animals do not produce cellulase on their own. These

microorganisms, however, are also capable of breaking down plant proteins and utilizing the released amino acids for their own growth. These ruminant microbes are subsequently digested as they pass through rest of the digestive tract of the animal, thereby providing an important source of protein and other nutrients. However, a large part of these proteins are deminated in the nitrogen excreted in the urine. Not only does this reduce the nutritional quality of the feed material, it also results in excess nitrogenous environmental pollution. The problem is exacerbated when ruminant animals are given very high protein diets in an effort to maximize milk production. Amino acid supplements (*e.g.*, methionine or lysine) are also subject to substantial degradation in the rumen and, thus, various rumen-protected amino acid supplements have been developed. Thus, it would be extremely desirable to feed intact proteins which are more resistant to microbial degradation. Therefore, it was important to determine if the zein proteins can be digested by the rumen bacteria and to determine if the zein proteins are digestible by the enzymes in the stomach of the ruminant animals.

Plant tissue was processed using mortar and pestle. Samples were placed into DACRON polyester bags (pore size 52 μm). Approximately .3 g of each sample was kept for comparison purposes and the remaining amount was incubated inside the rumen of a cannulated Holstein cow for a period of 12 hours. Bags were then removed from the rumen, washed and dried in a 60 degree C oven overnight. The 15kD zein and 10kD zein proteins were monitored immunologically (Western blotting) and by staining procedures. Ribulose biphosphate carboxylase which is highly degradable by ruminal bacteria (Nugent *et al.*, 1983) was monitored and used as a internal control. Very low levels of zein degradation was observed, whereas the ribulose biphosphate carboxylase was completely degraded after the treatment. The levels of zeins in the treated and untreated samples was determined to be comparable. It was also determined that the zein protein is digested by stomach enzymes of ruminant animals.

Example 9 – Induction of BiP in transgenic plants expressing the zein genes

Since BiP, an endogenous plant protein, has been implicated to have a role in prolamin protein body biogenesis (Li *et al.*, 1993a; Zhang and Boston, 1991), it followed that BiP may have a role in the formation of zein protein bodies in transgenic tobacco plants. To test whether BiP is increased in plants making zein protein bodies, protein samples from leaves of δ -zein, β -zein and δ -/ β -zein plants were subjected to quantitative western analysis using a corn BiP antibody (Zhang and Boston, 1992). The PBS soluble sample (100 μg) from a control plant and three transgenic plants (δ -, β -, and δ -/ β -zein plants) at the same developmental stage, all grown under the same conditions, along with 1 μg of purified BiP from corn, were subjected to SDS

PAGE followed by western analysis (Figure 8, top panel). The immunoreactive bands were then analyzed using the Intelligent Quantifier. The lower panel in Figure 8 is the graphical representation of the relative intensity of the immunoreactive bands. All three transgenic plants showed a significantly higher level of BiP accumulation when compared to the control; the levels of BiP were more or less similar in the three transformants. Thus, the synthesis of the zein proteins in the transgenic plants induces the synthesis or stable accumulation of BiP.

Example 10 – Expression and Stabilization of Proteins for the Purpose of Plant Pest Control

A chimeric gene (Z10/OCT) consisting of a 10kD zein gene (Z10) fused in frame to the front of an oryzacystatin I (OCI) protease inhibitor gene was designed to enhance the stability and effectiveness of OCI in transgenic plants for use in the control of plant pests. The chimeric gene includes both the coding and signal peptide regions of the 10kD zein and the coding region of the OCI, and is controlled by the constitutive CaMV 35S promoter. An anticipated protein product of approximately 26kD was expressed in plants transformed with the chimeric gene, as demonstrated on western blots probed with both OCI and 10kD zein antibodies. We have previously shown that plants transformed with 10kD zein, alone, express an expected protein product when probed with zein antibodies, while plants transformed with OCI, alone, show no detectable expression when probed with OCI antibodies. This observation shows that the Z10/OCI chimeric gene results in an increased stabilization of OCI, and further demonstrates the considerable stabilization of protein product by virtue of the discovery that zeins have a tendency to localize in enclosed protein bodies. Others have reported the effectiveness of protease inhibitors against various plant pests, including Colorado potato beetle and plant parasitic nematodes.

Example 11 – Construction of Transgenic Plants Expressing the 10 kD Zein\OCI Fusion Protein

The 10kDsp10kDzeinOC-1 (Z10/OCI) fusion was constructed using the spliced overlapping extension (SOE) technique (Horton, R.M. *et al.*, 1989). This technique utilizes the polymerase chain reaction (PCR) to splice together two separate fragments. The PCR was first used to amplify the Z10 fragment from plasmid prep (993) Z10pMON316/DH5 α using primers:

SEQ ID NO: 1 PA (CTACAAGATCTGATATCATCGATG) and

SEQ ID NO: 2 PB (GACATGGATCCGAATGCAGCAC)

to produce a product approximately 500 base pairs (bp) in length. The OC-1 fragment was amplified from plasmid (809) OclpSP73/DH5 α using primers:

SEQ ID NO: 3 PC (GTGCTGCATTTCGGATCCATGTCTG) and

SEQ ID NO: 4 PD (CCGGTACCCTTAAATCGATGC)

to produce a product of 322 bp fragment. The two PCR products were combined in equal concentrations and used as the template for the second PCR reaction. To obtain the desired Z10/OC1 fragment the primers, PA and PD, were used to produce a fragment of 800 bp. The
 5 bolded and larger nucleotides in the primer sequence PA, PB, and PC represent the sequences taken from the Z10 fragment. The smaller nucleotides in primer sequence PB, PC, and PD represent the sequence taken from Ocl. The bolded nucleotides are from the Z10 sequence and the smaller nucleotides are from the Ocl sequence. The primer sequences in Figure 9 are underlined and the primer name is in bold above or below the primer sequence. The underlined
 10 primer sequences PB and PD in Figure 9 are the reverse complement of the PB and PD sequence listing above. The orientation of the primer is indicated by arrows.

In order to clone this fragment into NewpFLAG-1 (NPF-1) the PCR fragment was made blunt ended by the addition of Klenow and dNTP's and ligated to NPF-1 digested with EcoRI and made blunt ended by the addition of Klenow and dNTP's. The Z10/OC1NPF-1 was ligated
 15 and transformed into DH5 α competent cells. Once the presence of the insert was confirmed by PCR the Z10/OC1NPF-1 was induced with IPTG and the induced protein was run on a Western blot. The protein samples were run in triplicate and probed with Z10, OC-1, and FLAG polyclonal antibodies. Reaction with all three antibodies proved that the entire construct was in frame. This Western blot data together with sequences analysis confirmed the accuracy of
 20 the nucleotide sequence. Sequence analysis was important to confirm that no PCR induced errors were introduced. However, before sequencing, the construct was digested with Bgl II and KpnI and ligated into similarly digested pSP73.

Example 12 – Tobacco Leaf Disc Transformation

25 In order to transform tobacco plants, the construct needed to be subcloned into plasmid pGG. Both the Z10/OC1NPF-1 and pGG were digested with Bgl II and Kpn I, ligated, and transformed into DH5 α . The presence of the insert was confirmed with PCR. Figure 10 is an overview of the construction of Z10/OC1. The plasmid pGG-2 is a derivative of pMON316 (Rogers *et al.*, 1987) with the addition of the AMV coat protein to enhance translation (Sutton,
 30 D.W. *et al.*, 1992). Triparental matings with pTiT37SE (Rogers *et al.*, *supra*) were used to co-integrate pGG-2 containing the Z10/OC1 insert. Tobacco leaf discs were transformed by the method of Horsch *et al.* (Horsch *et al.*, 1985). When individual tobacco shoots were visible they were transferred to Murashige and Skoog media (Murashige, T. and Skoog, F., 1962) containing clafaran (500 uf/ml) and kanamycin (100 ug/ml). The individual plants were then tested for

protein expression by Western blot.

Figures 11A and 11B are Western blots showing positive reaction with both the Z10 and OC1 antibodies.

- 5 It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

References

- Abe, S., W. You and E. Davis (1991) "Protein bodies in corn endosperm are enclosed by and enmeshed in F.-actin." *Protoplasma* **165**:139-149.
- 5 Argos, P., K. Pederson, M.D. Marks and B.A. Larkins (1982) "A structural model for maize zein proteins" *J. Biol. Chem.* **257**:9984-9990.
- Altenbach, S.B., C.C. Kua, L.C. Staraci, K.W. Pearson, C. Wainwright, A. Georgescu, and J. Townsend (1992) "Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine" *Pl. Mol. Biol.* **18**:235-245.
- 10 Aycock, M.K., Jr., and C.P. Wilsie (1967) "Inbreeding *Medicago sativa* L. by sibmating: Cross-, sib- and self-fertility" *Crop Sci.* **7**:281-284.
- 15 Bagga, S., Adams Hanke, J.D. Kemp, and C. Sengupta-Gopalan (1995) "Accumulation of the 15kD zein protein in novel protein bodies in transgenic tobacco" *Plant Physiol.* **107(1)**:13-23.
- 20 Bagga, S., Temple, S.J., Kemp, J.D. and Sengupta-Gopalan, C. (1997a) "A genetic engineering approach for improving protein quality of forage legumes" *Plant Molecular Biology and Biotechnology*, K.K. Tiwari and G.S. Singhal (eds.): Narosa Publishing House, pp. 305-319.
- 25 Bagga, S., Rodriguez, F., Adams, H., Kemp, J.D. and Sengupta-Gopalan, C. (1997b) "Co-expression of the maize α - and β -zein genes results in stable accumulation of the δ -zein in endoplasmic reticulum-derived protein bodies formed by β -zein" *Plant Cell* **9**:1683-1696.
- 30 Barnes, D.K., B.P. Goplen, and J.E. Baylor (1988) "Highlights in the USA and Canada" In: A.A. Hanson, D.K. Barnes, and R.R. Hill, Jr. (ed.) *Alfalfa and alfalfa improvement*. Agron. Magr. 29. ASA, Madison, WI.
- Bednarek, S.Y. and N.V. Raikhel. (1991) "The barley lectin carboxy-terminal propeptide is a vacuolar sorting determinant in plants" *Plant Cell* **3**:1195-1206.

- Bingham, E.T. and T.J. McCoy (1979) "Cultivated alfalfa at the diploid level: Origin, reproductive stability, and yield or seed and forage" *Crop Sci.* **19**:97-100.
- Bingham, E.T. (1983a) "Maximizing hybrid vigor in autotetraploid alfalfa. P. 130-143. In
5 Better Crops for Food. CIBA Found Symp. 97. Pitman Books, London.
- Bingham, E.T. (1983b) "Molecular genetic engineering vs. Plant breeding" *Plant Mol. Biol.* **2**:221-228.
- 10 Boston, R.S., E.B.P. Fontes, B.B. Shank and R.L. Wrobel (1991) "Increased expression of the maize immunoglobulin binding protein homology b-70 in three zein regulatory mutants" *Plant Cell* **3**:497-505.
- Cerioti, A., E. Pedrazzini, M. S. Fabbrini, M. Zoppe, R. Bollini and A. Vitale (1991)
15 "Expression of the wild type and mutated vacuolar storage protein phaseolin in xenopus oocytes reveals relationships between assembly and intracellular transport. *Eur. J. Biochem.* **202**:959-968.
- Chrispeels, M.J. (1991) "Sorting of protein in the secretory system" *Annu. Rev. Plant Physiol.*
20 *Plant Mol. Biol.* **42**:21-53.
- Chrispeels, M.J. and N.V. Raikhel (1992) "Short peptide domains target proteins to plant vacuoles" *Cell* **68**:613-616.
- 25 De Barros, E.G. and B.A. Larkins (1990) "Purification and characterization of zein degrading proteinases from germinating maize endosperm" *Plant Physiol.* **94**:297-303.
- DeClercq, A., M. Vandewele, R. De Rycke, J. Van Damme, M. Van aMontagu, E. Krebbers, J. Vandekerckjove (1990) "Expression and processing of an Arabidopsis 2S albumin in
30 transgenic tobacco" *Plant Physiol.* **94**:970-979.
- Dunbier, M.W., and E.T. Bingham (1975) "Maximum heterozygosity in alfalfa: Results using haploid-derived autotetraploids" *Crop Sci.* **15**:527-531.

- Edwards, G.A., A. Hepher, S.P. Clerk and D. Boulter (1991) "Pea lectin is correctly processed, stable and active in leaves of transgenic potato plants" *Plant Mol. Biol.* **17**:89-100.
- 5 Esen, A. and D.A. Stetter (1992) "Immunochemical location of γ -zein in the protein bodies of maize endosperm" *Am. J. Bot.* **79**:243-248.
- Finnegan, J. and D. McElroy (1994) "Transgene Inactivation: Plants fight back!" *Biotechnology* **12**:883-888.
- 10 Flavell, R.B. (1994) "Inactivation of gene expression in plants as a consequence of specific sequence duplication" *Proc. Natl. Acad. Sci. USA* **91**:3490-3496.
- Gallais, A. (1984) "An analysis of heterosis vs. Inbreeding effects with an autotetraploid cross-fertilized plant: *Medicago stiva* L" *Genetics* **106**:123-137.
- 15 Giannazza, E., V. Viglienghi, P.B. Righetti, F. Salamini, and C. Soave (1977) "Amino acid composition of zein molecular components" *Phytochemistry* **16**:315-317.
- Galili, G. (1995) "Regulation of lysine and threonine synthesis" *The Plant Cell* **7**:899-906.
- 20 Goldsbrough, P., B., S.B. Gelvin and B.A. Larkins (1986) "Expression of maize zein genes in transformed sunflower cells" *Mol. Gen. Genet.* **202**:374-381.
- Groose, R.W., W.P. Kojis, and E.T. Bingham (1988) "Combining ability differences between isogenic diploid and tetraploid alfalfa" *Crop Sci.* **28**:7-10.
- 25 Groose, R.W., L.E. Talbert, W.P. Kojis, and E.T. Bingham (1989) "Progressive heterosis in autotetraploid alfalfa: studies using two types of inbreds" *Crop Sci.* **29**:1173-1177.
- 30 Guerche, P., E.R.P. De Almeida, M.A. Schwarztein, E.S. Gander, E. Krebbers, and G. Pelletier (1990) "Expression of the 2S albumin from *Bertholletia excelsa* in *Brassica napus*" *Mol. Gen. Genet.* **221**:305-314.
- Hanson, A.A., D. K. Barnes, and R.R. Hill (1988) "Alfalfa and Alfalfa improvements"

Agronomy Monographs 29, ASA, CSSA and SSA, Madison Wisconsin USA.

Hilder, V.A., A.M.R., Gatehouse, S.E. Sheerman, R.F. Barker and D. Boulter (1987) "A novel mechanism of insect resistance engineered into tobacco" *Nature* **300**:160-163.

5

Hoffman, L.M., D.D. Donaldson, R. Bookland, K. Rashka, and E.M. Herman (1987) "Synthesis and protein body deposition of maize 15kD zein in transgenic tobacco seeds" *EMBO. J.* **6**:3213-3221.

10 Hoffman, L.M., Donaldson, and E.M. Herman (1988) "A modified storage protein is synthesized, processed and degraded in the seeds of transgenic plants" *Plant Mol. Biol.* **11**:717-729.

Horsch *et al.* (1985) *Science* **227**:1229-1231.

15

Horton, R.M. *et al.* (1989) *Gene* **77**:61-68.

Jorgensen, R.A. (1995) "Cosuppression, flower color patterns, and metastable gene expression states" *Science* **268**:686-691.

20

Kaldy, M.S., M.R. Hanna, S. Smoliak (1979) "Aminoacid composition of santoin forage" *Grass and Forage Sci.* **34**:145-148.

25

Karchi, H., O. Shaul and G. Galili (1993) "Seed-specific expression of a bacterial disensitized aspartate kinase increases the production of seed threonine and methionine in transgenic tobacco" *The Plant J.* **3**:721-727.

Kempthorne, O. (1969) "An introduction to genetic statistics" Iowa State University Press, Ames.

30

Kirhara, J.A., J.P. Hunsperger, W.C. Mahoney, and J.W. Messing (1988) "Differential expression of a gene for a methionine-rich storage protein in maize" *Mol. Gen. Genet.* **211**:477-484.

- Langridge, P. and G. Felix (1983) "A zein gene of maize is transcribed from two widely separated promoter regions" *Cell* **34**:1015-1022.
- 5 Larkins, B.A., C.R. Lending, J.C. Wallace, G. Galili, E.E. Kawata, K.B. Geetha, A.L. Kirz, D.M. Martin, and C.E. Bracker (1989) "Zein gene expression during maize endosperm development" In: Goldbert R.B. (Ed) The molecular basis of plant development, Alan R. Liss, NY pp. 109-120.
- 10 Larkins, B.A. and W.J. Hurkman (1978) "Synthesis and deposition of zein in protein bodies of maize endosperm" *Plant Physiol.* **62**:256-263.
- Lee, K.H., R.A. Jones, A. Dalby, and C.Y. Tsai (1976) "Genetic rgulation of storage protein content in maize endosperm" *Biochemical Genetics* **14**:641-650.
- 15 Lending, C.R. and B.A. Larkins (1989) "Changes in the zein composition of protein bodies during maize endosperm development" *Plant Cell* **1**:1011-1023.
- Levanony, H., R. Rubin and Galili G. Altschuler (1992) "Evidence for a novel rout of wheat storage proteins to vacuoles" *J. Cell Biol.* **119**:1117-1128.
- 20 Li, X., Franceshi, V.R. and T.W. Okita (1993a) "Segregation of storage protein mRNAs on the rough endoplasmic reticulum membranes of rice endosperm cells" *Cell* **72**:869-879.
- Li, X., Wu, Y., D-Z Zhang, J.W. Gillikin, R.S. Boston, V.R. Franceschi and T.W. Okita (1993b)
- 25 "Rice prolamine protein body biogenesis: a BiP-mediated process" *Science* **262**:1054-1056.
- Matsuoka, K., K. Nakamura (1991) "Propeptide of a precursor to a plant vacuolar protein required for vacuolar targeting" *PNAS USA* **88**:834-838.
- 30 Maliga, P. (1978) "Resistant mutant and their use in genetic manipulation" In: Frontiers of plant tissue culture (Ed: Thorpe TA) pp 381-392.
- Masumura, T., T. Hibino, K. Kidza, N. Mitsukawa, K. Tanaka and S. Fujii (1990) "Cloning and

characterization of a cDNA encoding a rice 13kD prolamin" *Mol. Gen. Genet* **221**:1-7.

- 5 Meyer, P. and I. Heidman (1994) "Epigenetic variants of a transgenic petunia line shows hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants" *Mol. Gen. Genet.* **243**:390-399.

- 10 Michaud, R., W.F. Lehman, and M.D. Rumbaugh (1988) "World distribution and historical development" In A.A. Hanson, D.K.J. Barnes, and R.R. Hill, Jr. (Ed) *Alfalfa and alfalfa improvement. Agron. Mongr. 29*, ASA, Madison, WI.

- Minson, D.J. (1990) Protein. In: Forage in Ruminant Nutrition, Academic Press Inc., New York).

- 15 Misra, P.S., R. Jumbunathan, E.T. Mertz, D.V. Glover, H.M. Barbosa, K.S. McWhirter (1972) "Endosperm protein synthesis in maize mutants with increased lysine content" *Science* **176**:1425-1426.

- Murashige, T., F. Skoog (1962) *Physiology Plant* **15**:485.

- 20 Nugent, J.H.A., W.T. Jones, D.J. Jones and J.L. Mangan (1983) "Rates of proteolysis in the rumen of the soluble proteins casein, Fraction I (18S) leaf protein bovine serum and bovine submaxillary mucoprotein" *Br. J. Nutr.* **50**:357-368.

- 25 Ohtani, T., J.C. Wallace, G.A. Thompson, G. Galili, and B.A. Larkins (1990) "Normal and lysine containing zeins are unstable in transgenic tobacco seeds" *Plant Mol. Biol.* **15**:117-128.

- Pelham, H.R.B. (1990) "The retention signal for soluble proteins of the endoplasmic reticulum" *Trends Biochem Sci.* **15**:483-485.

- 30 Phillips, R.L., B.A. McClure (1985) "Elevated protein bound methionine in seeds of maize line resistant to lysine plus threonine cereal chem. *62*:213-218.

- Rogers, et al. (1987) *Methods of Enzymology* **153**:253-277.

Sutton, D.W. *et al.* (1992) *Transgenic Research* **1**:228-236.

Tabe, L.M., Higgins, C.M., McNabb, W.C. and Higgins, T.J.V. (1993) "Genetic engineering of grain and pasture legumes for improved nutritive value" *Genetica* **90**:181.

Claims

- 1 1. A plant or plant tissue comprising a storage protein that is expressed and accumulated
2 as a protein body in a vegetative tissue of said plant or plant tissue.
- 1 2. The plant or plant tissue, according to claim 1, wherein said storage protein is rumin
2 stable.
- 1 3. The plant or plant tissue, according to claim 2, wherein said rumin stable protein is
2 a zein protein.
- 1 4. The plant or plant tissue, according to claim 3, wherein said zein is selected from the
2 group consisting of the 15kD zein and 10kD zein.
- 1 5. The plant or plant tissue, according to claim 1, wherein said plant co-expresses both
2 the 15kD and 10kD zein proteins.
- 1 6. The plant or plant tissue, according to claim 1, wherein said storage protein is
2 constitutively expressed in said plant or plant tissue.
- 1 7. A method for increasing the forage quality of a plant, comprising transforming a plant
2 or plant tissue with a polynucleotide molecule that encodes a storage protein that is expressed
3 and accumulated as a protein body in a vegetative tissue of said plant or plant tissue.
- 1 8. The method, according to claim 7, wherein said storage protein is rumin stable.
- 1 9. The method, according to claim 8, wherein said rumin stable protein is a zein protein.
- 1 10. The method, according to claim 9, wherein said zein is selected from the group
2 consisting of the 15kD zein and 10kD zein.
- 1 11. The method, according to claim 7, wherein said plant co-expresses both the 15kD
2 and 10kD zein proteins.

1 12. A method for increasing the stability and storage of a heterologous protein in a plant,
2 comprising transforming a plant or plant tissue expressing a heterologous protein with a
3 polynucleotide molecule that encodes a storage protein that is expressed and accumulated as a
4 protein body in a vegetative tissue of said plant or plant tissue.

1 13. The method, according to claim 12, wherein said storage protein is rumin stable.

1 14. The method, according to claim 13, wherein said rumin stable protein is a zein
2 protein.

1 15. The method, according to claim 14, wherein said zein is selected from the group
2 consisting of the 15kD zein and 10kD zein.

1 16. The method, according to claim 12, wherein said plant co-expresses both the 15kD
2 and 10kD zein proteins.

1 17. A composition comprising a rumin stable protein body, wherein said protein body
2 is expressed and accumulated in a plant.

1 18. The composition, according to claim 17, wherein said protein body comprises a zein
2 protein.

1 19. The composition, according to claim 18, wherein said zein protein is selected from
2 the group consisting of the 15kD and 10kD zein.

1 20. The composition, according to claim 17, wherein said protein body comprises the
2 15kD and 10kD zein.

SEQUENCE LISTING

<110> New Mexico State University Technology Transfer Corporation

120> Co-Expression of Proteins

<130> MPS-410C2

<140> 09/224,655

<141> 1998-12-31

<150> 08/866,879

<151> 1997-05-30

<150> 60/020/424

<151> 1996-05-30

<160> 6

<170> PatentIn Ver. 2.0

<210> 1

<211> 24

<212> DNA

<213> Unknown

<220>

<223> Description of Unknown Organism: Synthetic

<400> 1

ctacaagatc tgatatcatc gatg

24

<210> 2

<211> 22

<212> DNA

<213> Unknown

<220>

<223> Description of Unknown Organism: Synthetic

<400> 2

gacatggatc cgaatgcagc ac

22

<210> 3

<211> 23

<212> DNA

<213> Unknown

<220>

<223> Description of Unknown Organism: Synthetic

<400> 3

gtgctgcatt cggatccatg tcg

23

<210> 4

<211> 21

<212> DNA

<213> Unknown

<220>

<223> Description of Unknown Organism: Synthetic

<400> 4

ccggtaccct taaatcgatg c

21

<210> 5

<211> 444

<212> DNA

<213> Zea mays

<400> 5

agatctgtat catcgatgaa ttccggaagc aaggacacca ccgccatggc agccaagatg 60

```
catattccag ggcacttgcc accagtcacg ccattgggta ccatgaaccc atgcatgcag 120
tactgcatga tgcaacaggg gcttgccagc ttgatggcgt gtccgtccct gatgctgcag 180
caactgtttg ccttaccgct tcagacgatg ccagtgatga tgccacagat gatgacgcct 240
aacatgatgt caccattgat gatgccgagc atgatgtcac caatgggtctt gccgagcatg 300
atgtcgcaaa tgatgatgcc acaatgtcac tgcgacgccg tctcgagat tatgctgcaa 360
cagcagttac cattcatgtt caaccaatg gccatgacga ttccacccat gttcttacag 420
caaccctttg ttggtgctgc attc                                     444
```

<210> 6

<211> 322

<212> DNA

<213> Oryza sativa

<400> 6

```
ggatccatgt cgagcgacgg agggccggtg cttggcggcc tcgagccggt ggggaacgag 60
aacgacctcc acctcgctga cctcgcccg ctcgccgtca ccgagcacia caagaaggcc 120
aattccctgc tggagtgcga gaagcttggt agtgtgaagc agcaagttgt cgctggcact 180
ttgtactatt tcacaattga ggtgaaggaa ggggatgcc aagaagctcta tgatgctaag 240
gtctgggaga aaccatggat ggacttcaag gagctccagg agttcaagcc tgcgatgcc 300
tcggcatcga ttttaagggt cc                                     322
```

1/17

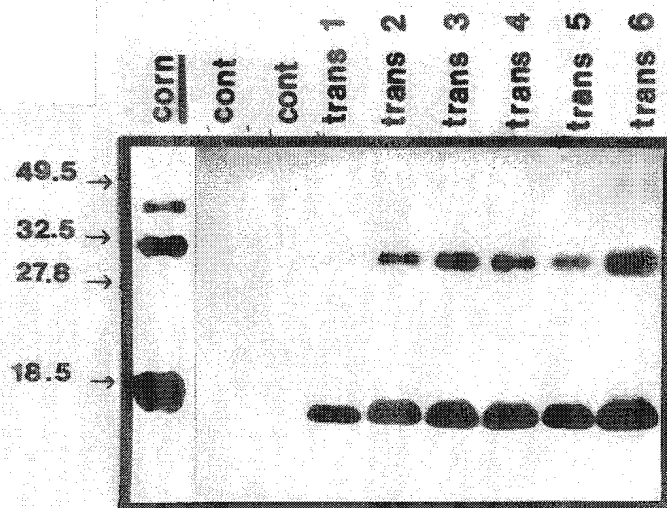


FIG. 1A

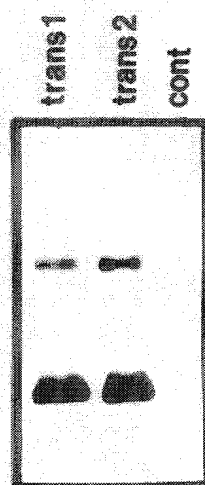


FIG. 1B

2/17

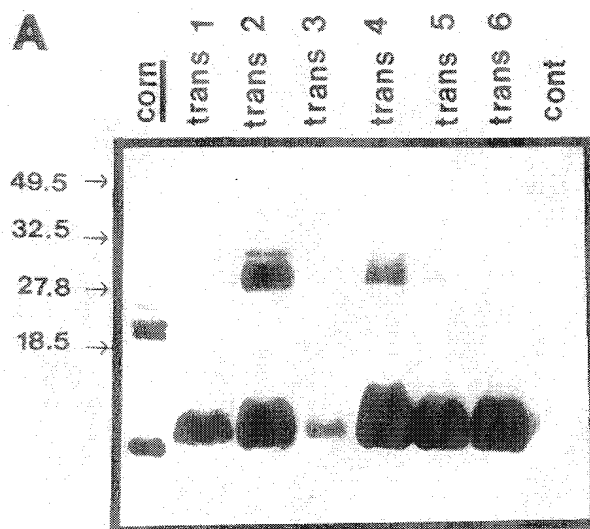


FIG. 2A

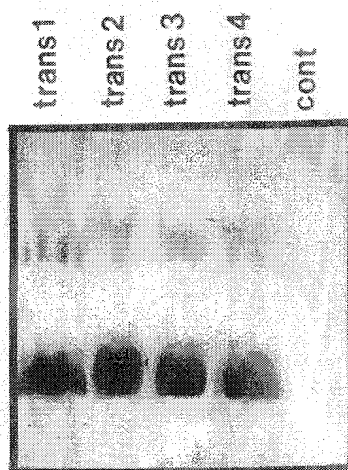


FIG. 2B

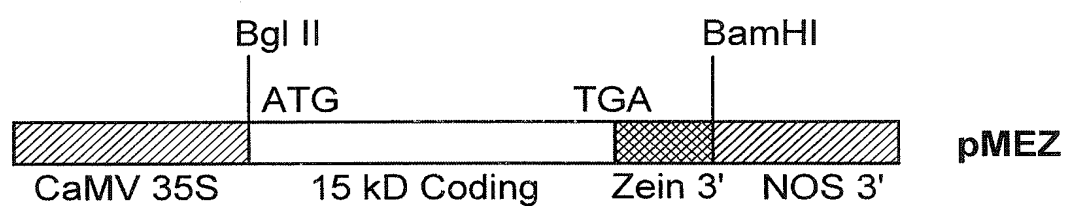


FIG. 1C

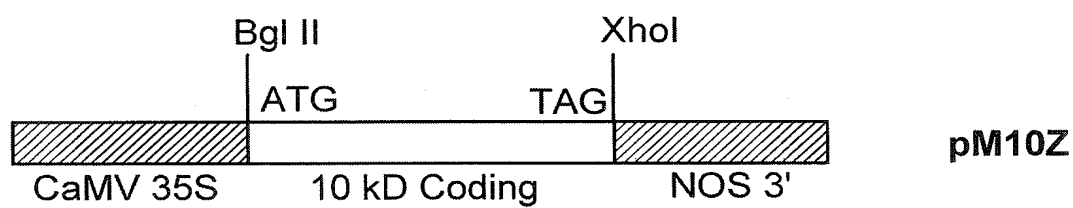


FIG. 2C

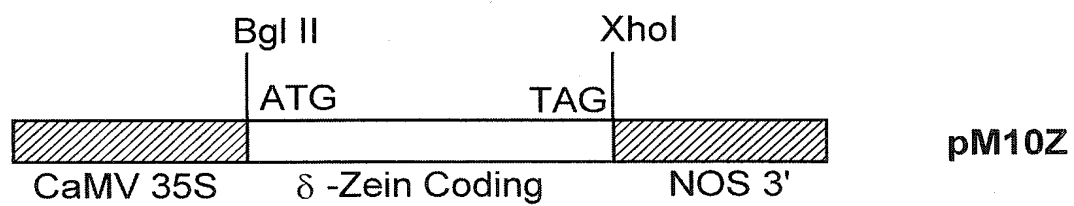


FIG. 3A

5/17

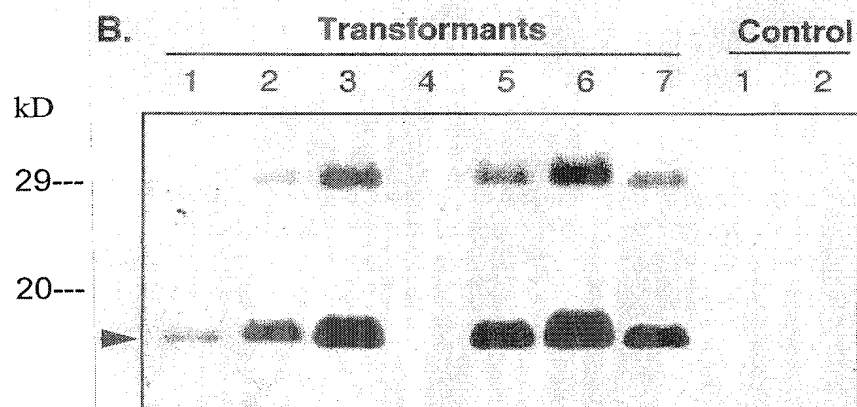


FIG. 3B

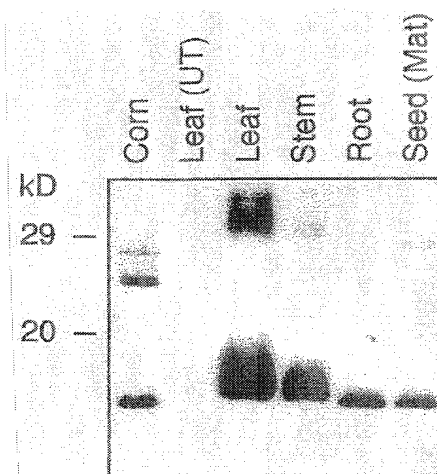


FIG. 3C

6/17

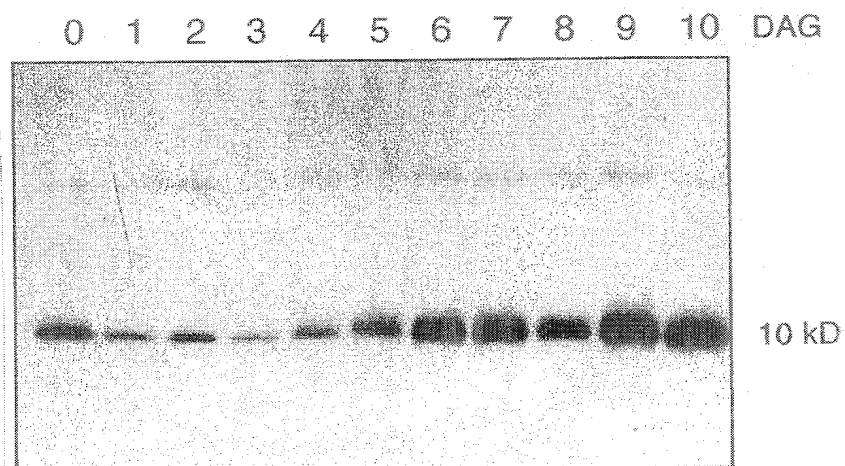


FIG. 4

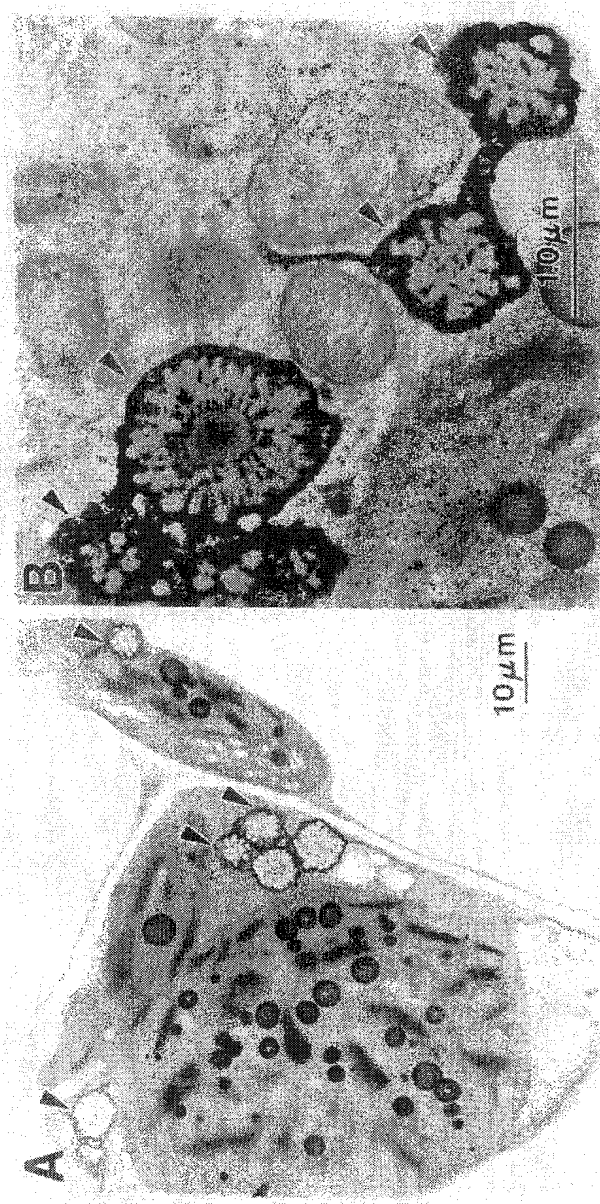


FIG. 5B

FIG. 5A



FIG. 5D

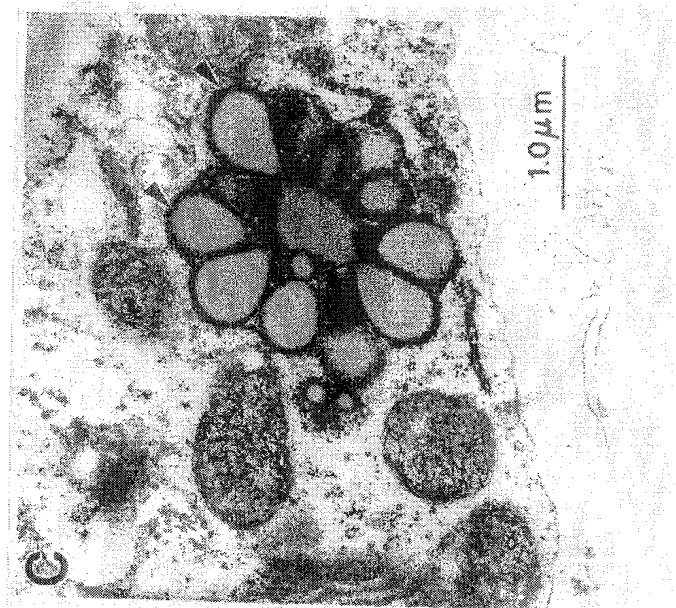


FIG. 5C

9/17

FIG. 6A

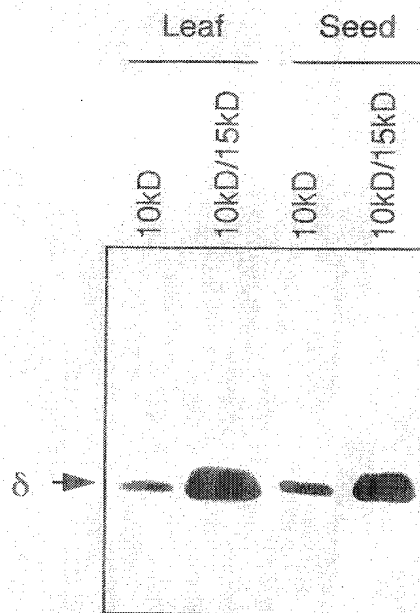
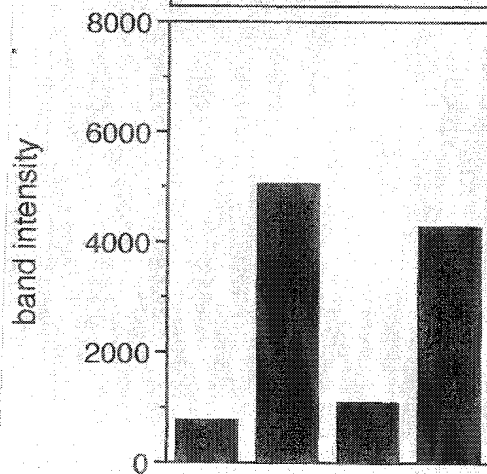


FIG. 6B



10/17

FIG. 6C

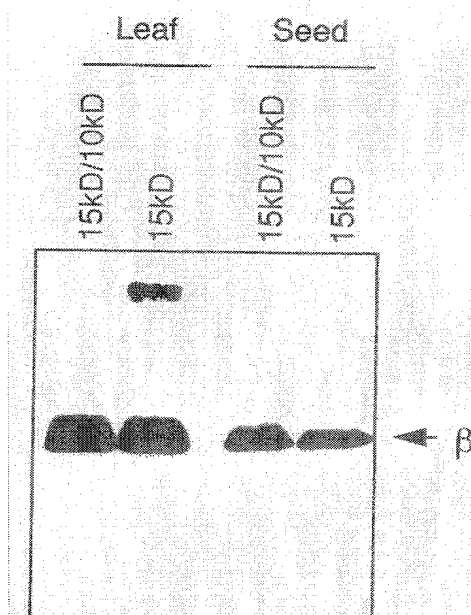
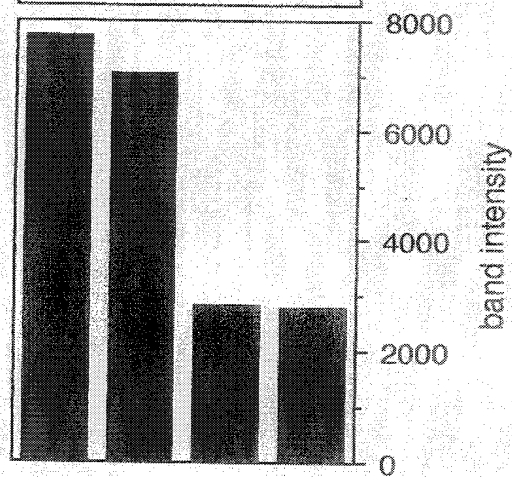
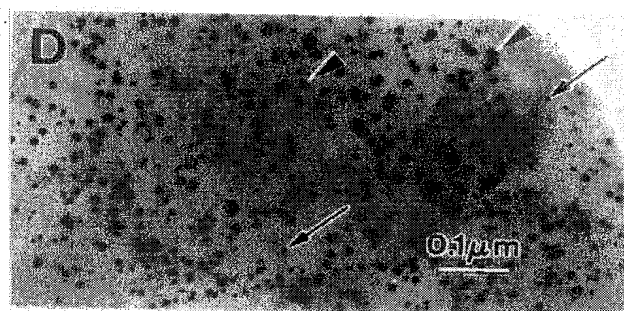
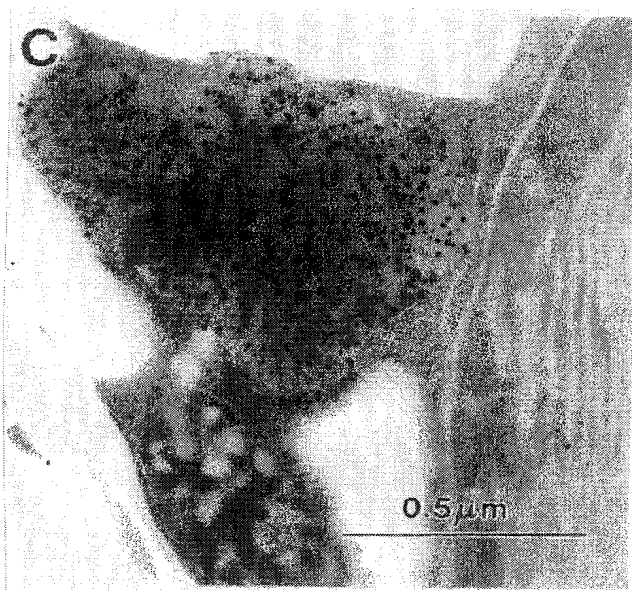
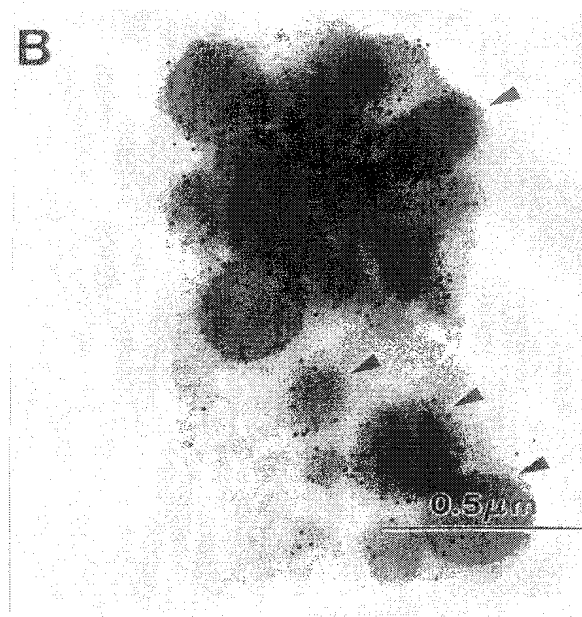
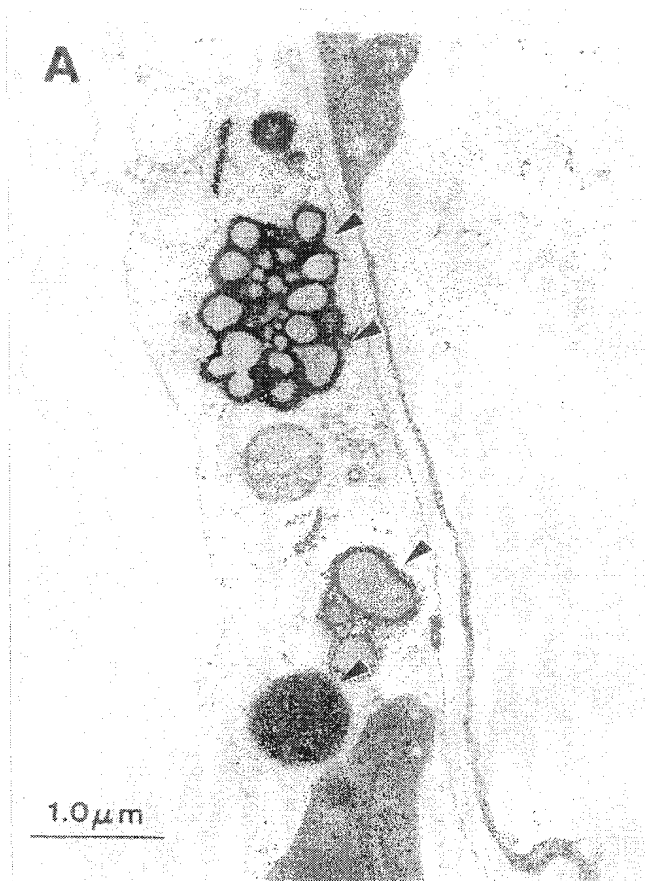


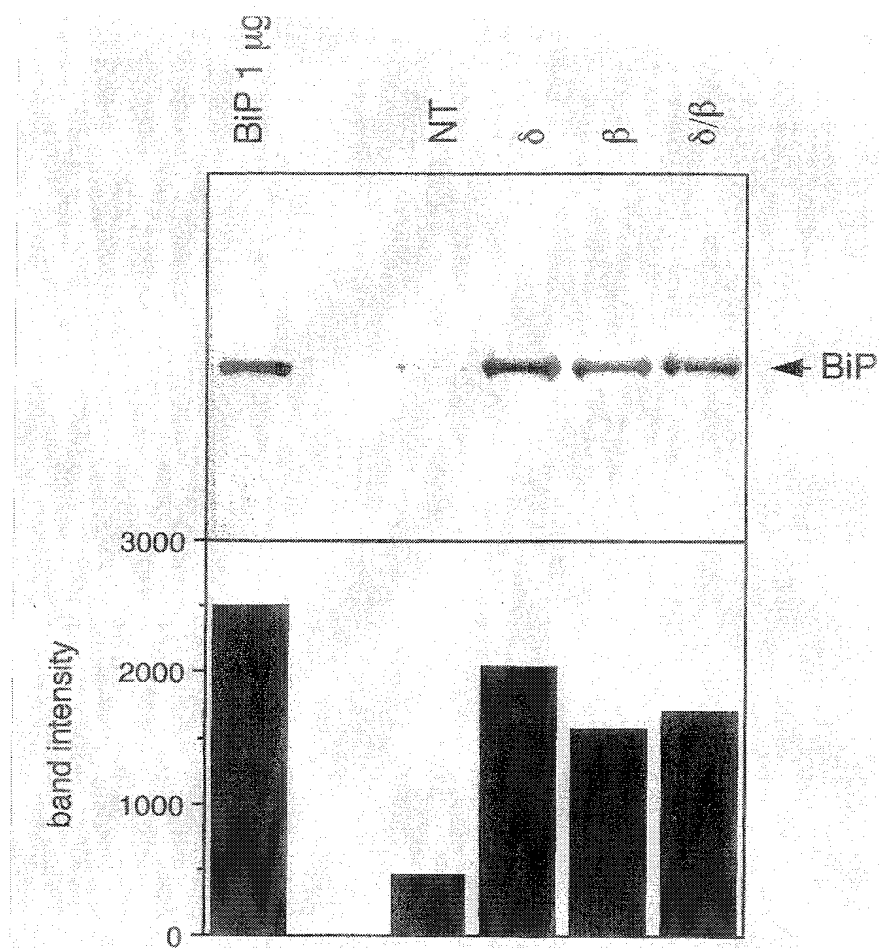
FIG. 6D





12/17

FIG. 8.



13/17

10 20 30 40 50 60
PA →
1 AGATCTGTAT CATCGATGAA TTCCGGAAGC AAGGACACCA CCGCCATGGC AGCCAAGATG
61 CTGCAATTGT TCGCTCTCCT AGCTCTTTGT GCAAGCGCCA CTAGTGGATC TATGGCGACC
121 CATATTCCAG GGCACCTTGCC ACCAGTCATG CCATTGGGTA CCATGAACCC ATGCATGCAG
181 TACTGCAATGA TGCAACAGGG GCTTGCCAGC TTGATGGCGT GTCCGTCCCT GATGCTGCAG
241 CAACTGTTGG CCTTACCGCT TCAGACGATG CCAGTGATGA TGCCACAGAT GATGACGCCT
301 AACATGATGT CACCATTGAT GATGCCGAGC ATGATGTCAC CAATGGTCTT GCCGAGCATG
361 ATGTCGCAA TGAATGATGCC ACAATGTCAC TGCAGCGCCG TCTCGCAGAT TATGCTGCAA
421 CAGCAGTTAC CATTCAATGTT CAACCCAATG GCCATGACGA TTCCACCCAT GTTCTTACAG
481 CAACCCCTTG TTGGTGCTGC ATTC
←PB

FIG. 9A

14/17

10 20 30 40 50 60
PC → | | | | |
1 GGATCCATGT CGAGCGACGG AGGGCCGGTG CTTGGCGGCC TCGAGCCGGT GGGGAACGAG |
61 AACGACCTCC ACCTCGTCGA CCTCGCCCGC TTGCGCGTCA CCGAGCACAA CAAGAAGGCC
121 AATTCCTGC TGGAGTTCGA GAAGCTTGTG AGTGTGAAGC AGCAAGTTGT CGCTGGCACT
181 TTGTACTATT TCACAATTGA GGTGAAGGAA GGGGATGCCA AGAAGCTCTA TGATGCTAAG
241 GTCTGGGAGA AACCATGGAT GGACTTCAAG GAGCTCCAGG AGTTCAAGCC TGTCGATGCC
301 TCGGCATCGA TTTAAGGGTA CC ← PD

FIG. 9B

15/17

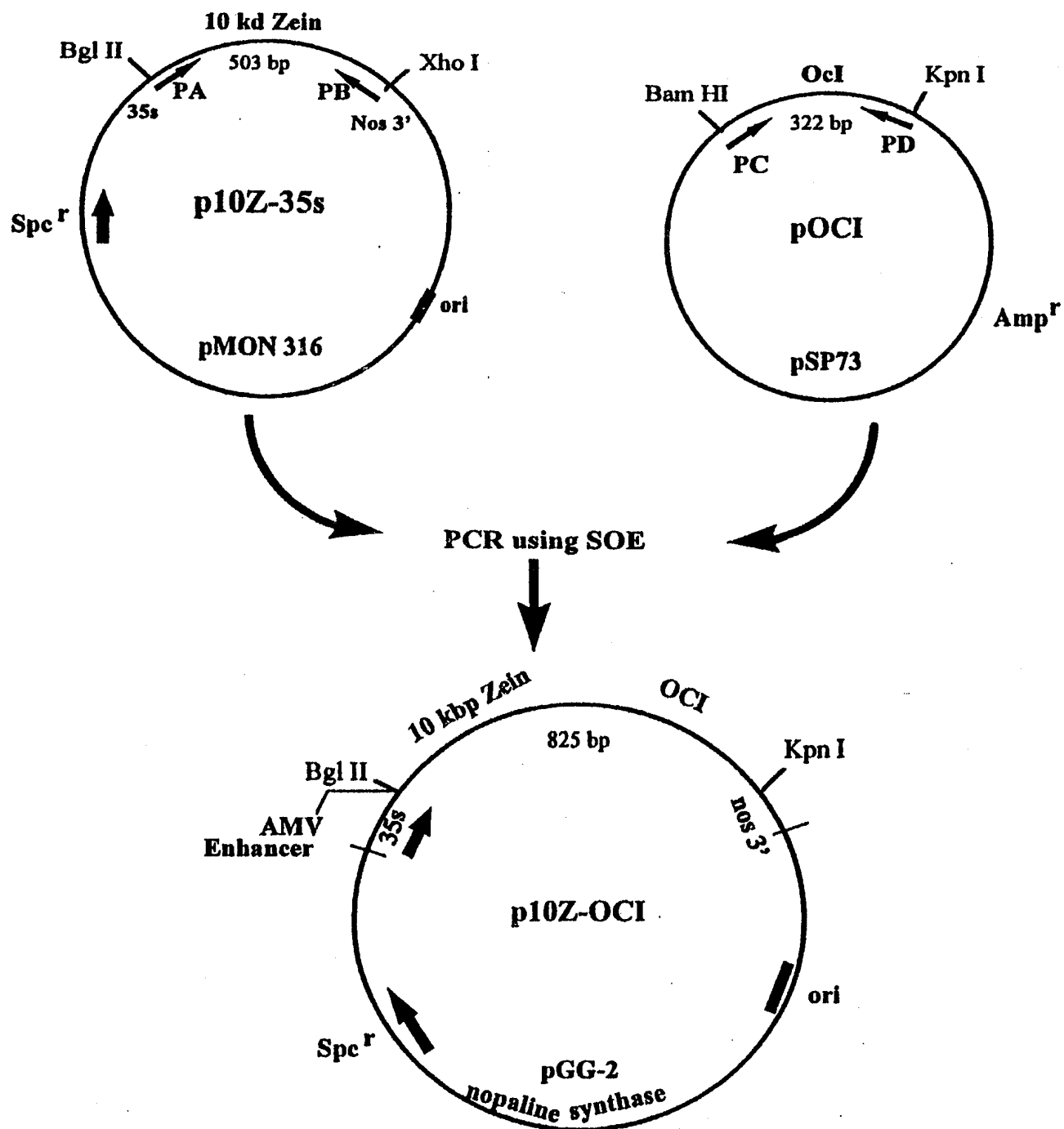


FIG. 10
SUBSTITUTE SHEET (RULE 26)

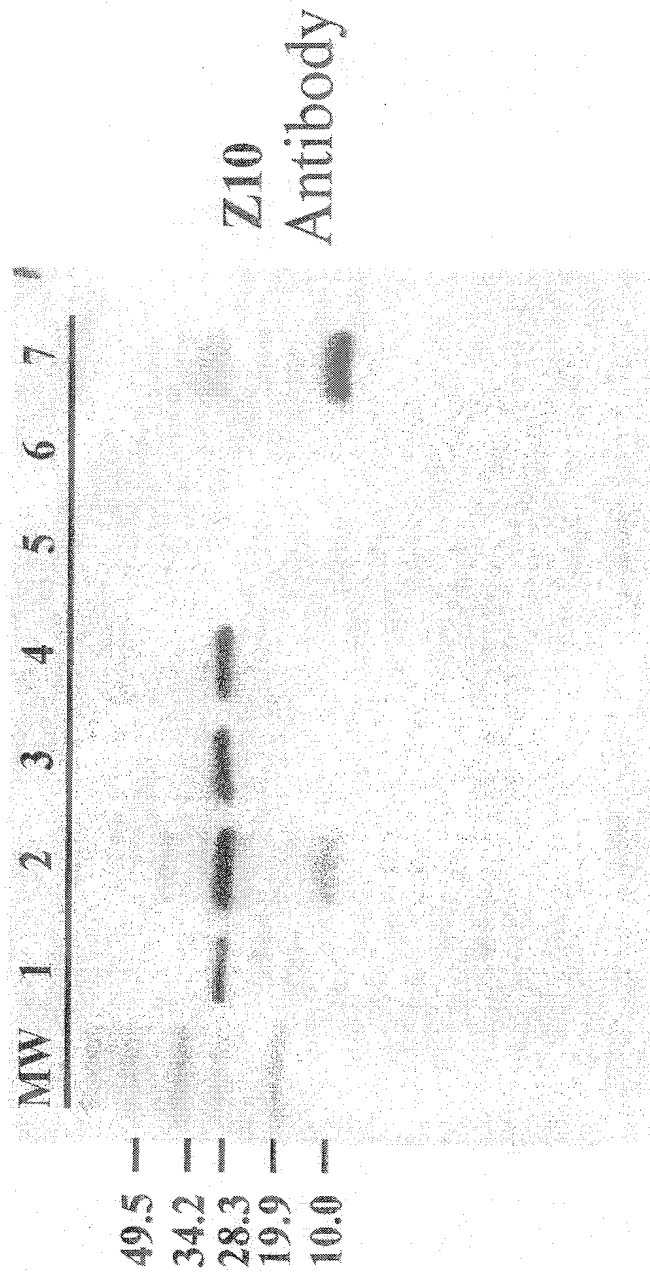


FIG. 11A

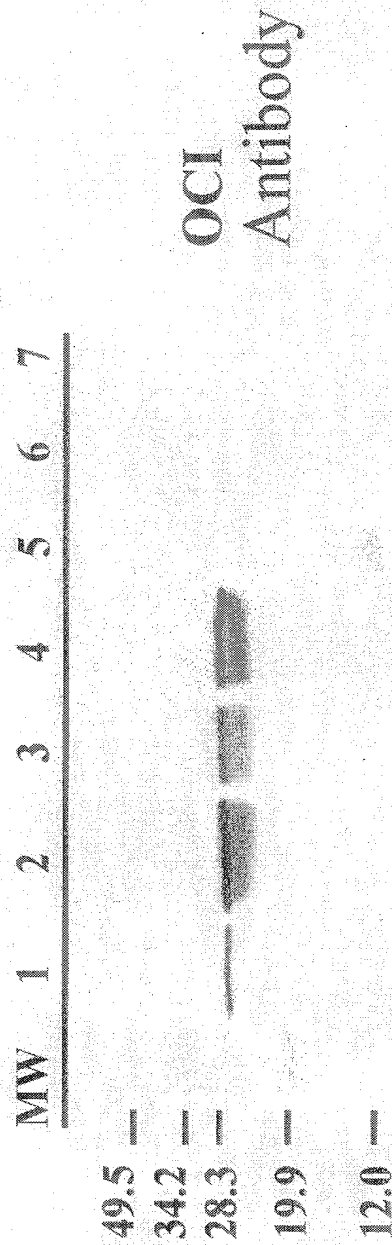


FIG. 11B

INTERNATIONAL SEARCH REPORT

Inter. Application No

PCT/US 99/08525

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C07K14/425 C12N15/29 C12N15/62 C07K16/16
C07K16/38 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BAGGA, S., ET AL.: "genetic engineering for enhancing the sulphur amino acids in forage legumes" SUPPLEMENT TO PLANT PHYSIOLOGY, vol. 111, no. 2, June 1996 (1996-06), page 166 XP002077183	1,6,7,12
Y	the whole document	2-4, 8-10, 13-15, 17-19
	--- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

10 January 2000

Date of mailing of the international search report

18/01/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Holtorf, S

INTERNATIONAL SEARCH REPORT

Inter- national Application No

PCT/US 99/08525

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BAGGA S ET AL: "ACCUMULATION OF 15-KILODALTON ZEIN IN NOVEL PROTEIN BODIES IN TRANSGENIC TOBACCO" PLANT PHYSIOLOGY, vol. 107, 1995, pages 13-23, XP002070069	1,6,7,12
Y	the whole document	2-4, 8-10, 13-15, 17-19
X	--- GELI M I ET AL: "TWO STRUCTURAL DOMAINS MEDATE TWO SEQUENTIAL EVENTS IN GAMMA-ZEIN TARGETING: PROTEIN ENDOPLASMIC RETICULUM RETENTION AND PROTEIN BODY FORMATION" PLANT CELL, vol. 6, no. 12, December 1994 (1994-12), pages 1911-1922, XP002017847	1,6,7,12
Y	abstract, page 1991, right column; page 1912, right column; Fig. 2B; page 1914, right column; Fig. 5; page 1918 right column - page 1919	2-4, 8-10, 13-15, 17-19
X	--- BAGGA, S., ET AL.: "coexpression of maize delta-Zein and beta-Zein genes results in stable accumulation of delta-Zein in endoplasmatic reticulum-derived protein bodies formed by beta-Zein" THE PLANT CELL, vol. 9, September 1997 (1997-09), pages 1683-1696, XP002077186	1,5-7, 11,12,16
Y	the whole document	2-4, 8-10, 13-15, 17-20
X	--- WO 97 28247 A (BIOCEM ;LUDEVID DOLORES (ES); TORRENT MARGARITA (ES); ALVAREZ INAK) 7 August 1997 (1997-08-07)	1,7,12
Y	page 2, line 3-6; page 4, line 22-27; page 9, line 24 - page 10, line 3; page 11, line 11; page 37-40; page 46; claim 18	2-4, 8-10, 13-15, 17-19
Y	--- HANCOCK, K.R., ET AL.: "identification of sulphur-rich proteins which resist rumen degradation and are hydrolysed rapidly by intestinal proteases" BRITISH JOURNAL OF NUTRITION, vol. 72, no. 6, December 1994 (1994-12), pages 855-863, XP002077184	2-4, 8-10, 13-15, 17-20
	the whole document	

	-/--	

INTERNATIONAL SEARCH REPORT

Inter nal Application No

PCT/US 99/08525

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>COLEMAN, C.E., ET AL.: "the maize gamma-Zein sequesters alpha-Zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm" THE PLANT CELL, vol. 8, December 1996 (1996-12), pages 2335-2345, XP002077185 the whole document</p> <p>-----</p>	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/08525

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9728247 A	07-08-1997	FR 2744134 A	01-08-1997
		AU 1550297 A	22-08-1997
		CA 2242903 A	07-08-1997
		EP 0877754 A	18-11-1998
